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USSR Report

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BIOMEDICAL AND BEHAVIORAL SCIENCES

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CONTENTS

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ANTHRAX INCIDENCE IN WORLD (MORBIDITY RATE IN FOREIGN COUNTRIES)

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 (manuscript received 15 Feb 83) pp 3-7

[Article by B. L. Cherkasskiy, Central Epidemiology Scientific Research Institute of the USSR Ministry of Health, Moscow]

[Text] The scientific literature contains descriptions of the incidence of anthrax in the world for 1944-1954 [15], 1950-1952 [4], 1951-1956 [1], 1959-1961 [12], 1955-1964 [2], and 1961-1970 [3]. In the present work we wish to discuss the incidence of anthrax in the world over the past decade (1971-1980) and to offer a brief epidemiological description of this infection on various continents.

It is difficult to evaluate the incidence of anthrax in the world using only official data from WHO [World Health Organization] because the data are incomplete and many countries submit information on an irregular basis. Nonetheless, WHO data and the literature available to us allow us to describe the basic trends in the dynamics of morbidity and several epidemiological peculiarities of the infection in various parts of the world.

It should be pointed out right away that in the majority of countries that have had the most trouble with anthrax a mass vaccination campaign among livestock is being carried out now against this infection, while humans are not, as a rule, immunized because there are no effective medical vaccines.

In European countries the relative incidence of anthrax among humans increases as one moves from north to south (table 1). There is either no incidence, or only sporadic recording of the disease in Northern European countries that are above 50° north latitude (the Scandinavian states, Great Britain, Iceland, Denmark, etc.) Incidence of the infection is recorded sporadically, but more frequently, in Central European countries located between 45° and 50° north latitude (the FRG, GDR, Hungary, Czechoslovakia, etc.) There is a higher morbidity recorded every year in Southern European countries located further south than 50° north latitude (Greece, Spain, Italy, Portugal, Romania, Yugoslavia, etc.) In spite of the fact that in the last decade as a result of mass vaccination there was a significant decrease in the incidence of anthrax among humans in the majority of European countries, it maintained its previous geograpical distribution: the maximum incidence was recorded in Southern Europe and the minimum incidence was recorded in Northern Europe.

Table 1. Incidence of anthrax among humans in European countries between 1967 and 1977 (based on WHO data)

Country	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
Австрия (1)	2	1	2	—	—	—	1	—	—	—	—
Бельгия (2)	—	4	1	1	—	1	2	—	1	—	1
Болгария (3)	59	57	39	49	36	28	126	33	26	23	21
Англия и Уэльс (4)	19	9	3	5	4	4	1	2	4	4	.
Северная Ирландия (5)	2	—	—	—	—	—	—	—	—	—	.
Шотландия (6)	5	1	8	2	1	1	—	3	.	—	.
Венгрия (7)	10	27	4	5	10	4	6	4	—	4	—
ГДР (8)	.	.	.	.	.	.	1	—	.	4	1
ФРГ (9)	8	3	3	2	6	3	3	4	6	2	3
Греция (10)	118	102	93	95	56	43	43	55	99	59	69
Испания (11)	316	350	311	284	198	220	198	232	251	216	.
Италия (12)	143	153	108	78	83	75	74	69	60	.	.
Норвегия (13)	1	—	—	—	—	—	—	—	—	—	—
Польша (14)	5	10	3	3	11	4	3	4	—	.	.
Португалия (15)	29	37	7	10	10	8	3	3	.	9	10
Румыния (16)	132	150	133	94	95	119	131	96	86	43	36
Финляндия (17)	—	—	—	—	—	—	—	—	—	—	—
Франция (18)	—	—	4	1	3	1	—	1	3	1	.
Чехословакия (19)	8	7	.	—	.	.	.	.	.	—	1
Швейцария (20)	—	—	—	—	—	1	1	.	.	—	1
Швеция (21)	1	—	—	—	—	—	—	—	—	—	.
Югославия (22)	153	130	105	84	85	56	57	.	.	.	.

Note: Here and in tables 2-4 the symbol - indicates that there was no incidence of the disease; the symbol . indicates that no data were available.

Key:

1. Austria
2. Belgium
3. Bulgaria
4. England and Wales
5. Northern Ireland
6. Scotland
7. Hungary
8. GDR
9. FRG
10. Greece
11. Spain
12. Italy
13. Norway
14. Poland
15. Portugal
16. Romania
17. Finland
18. France
19. Czechoslovakia
20. Switzerland
21. Sweden
22. Yugoslavia

In our opinion, these differences are determined by a complicated set of factors: the primary development of pasture livestock breeding has occurred in the southern part of the continent in combination with soil and climatic conditions that are favorable for preserving the infectious agent in the external environment; this is in addition to the relatively low level of economic and cultural development and organization of veterinary and sanitary preventive measures in Southern European capitalist countries compared to that in Northern European countries.

Under similar natural and geographic conditions, the differences in the level and rate of decline in the incidence of anthrax among humans are determined by

social and economic factors, which can be graphically illustrated by comparing the situations in Greece and Bulgaria. As a result of mass immunization of livestock in both these countries, there was a signficant decline in the incidence of anthrax among humans: in Greece the average incidence of the disease in 1975-1980 was 33, which was about one-fourth the average incidence recorded for 1960-1965. In Bulgaria, the annual incidence dropped from 219 to 27 cases, which represents a decrease to about one-eighth the previous level. The more rapid decline in the incidence of the disease in Bulgaria, in addition to the vaccination of the livestock, was accompanied by the creation of agro-industrial complexes, keeping livestock in stalls, and utilization of pastures not favorable for sowing grain crops.

In different parts of the European continent anthrax is characterized by certain epidemiological features. In Southern European countries humans are infected primarily through direct contact with infected animals in the process of tending them, slaughtering them, cutting up carcasses, and skinning them; while in Northern and Central European countries the infection of humans is primarily occupational in nature and is tied to livestock products, mainly those imported from countries of Asia, Africa, and South America that have particular problems with this disease. The most common factor for the spread of the infectious agent in these cases is the animal hides, as well as artificial livestock feed that contains bone, meat and blood meal. Cases of humans being infected by imported meat and bone meal have been reported a number of times in Great Britain [7, 9], when the meal was imported from Lebanon, Pakistan, and Argentina; such cases have also been reported in Belgium [8] and other European countries. In Dunkirk (France) four cases were reported in which port workers who unloaded a commercial vessel carrying infected bone meal from India were stricken with the disease [10].

On the American continent the most infected countries are countries in South America with intensive animal husbandry: Chile, Uruguay, Paraguay, Argentina, Peru, and El Salvador (table 2). Haiti and Guatemala are the Central American countries with the worst incidence of anthrax.

In countries of South and Central America livestock that has not been inoculated becomes infected with the disease in contaminated pastures and humans become infected with anthrax through direct contact with infected animals. This type of morbidity is also characteristic of Mexico.

In Canada and the United States there is immunization of livestock but there are still outbreaks of anthrax among agricultural animals recorded in various years. For example, in the southeast part of the state of Louisiana in 1971 anthrax was responsible for the death of 636 cows, 31 horses, and 33 other animals [11]; in June and July of 1974 in Falls County, Texas, 26 herds were stricken with anthrax. Cases of anthrax among humans occur only sporadically (between 1967 and 1978 2-6 cases per year were reported in the United States) and these cases involve primarily industrial workers who have been contaminated by imported raw materials of animal origin. Of the 6 cases of anthrax in humans reported in the United States in 1978, 4 (2 in North Carolina and 2 in New Hampshire) occurred at textile and felt plants [6].

Table 2. The incidence of anthrax among humans in American countries between 1967 and 1977 (based on WHO data)

Страна Country	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
Аргентина (1)	168	42	71	64	120	89	111	46	39	57	81
Венесуэла (2)	8	—	—	2	2	11	—	—	—	.	.
Гаити (3)	17	66	200	23	48	41	58	.	66	62	73
Гватемала (4)	.	.	.	.	.	34	18	5	.	15	.
Канада (5)	—	—	—	1	—	—	—	—	—	—	—
Колумбия (6)	5	3	18	7	.	—	6	2	.	.	.
Мексика (7)	25	24	35	22	29	20	11	10	.	10	14
Панама (8)	1	1	1	2	2	—	—	—	—	—	.
Парагвай (9)	8	2	26	25	30	20	50	.	.	.	.
Перу (10)	26	89	55	57	106	60	50	84	57	113	137
Пуэрто-Рико (11)	.	.	.	.	—	—	—	—	—	.	.
Сальвадор (12)	61	26	16	34	20	35	16	1	7	2	2
США (13)	2	3	4	2	5	1	2	2	2	2	—
Уругвай (14)	45	58	37	22	27	23	23	16	5	10	.
Чили (15)	179	158	78	86	90	74	72	80	95	61	42

Key:

1. Argentina
2. Venezuela
3. Haiti
4. Guatelmala
5. Canada
6. Columbia
7. Mexico
8. Panama
9. Paraguay
10. Peru
11. Puerto Rico
12. El Salavdor
13. United States
14. Uruguay
15. Chile

Data on anthrax in Asia is incomplete, since there is no information on the incidence of anthrax among the populations of large and densely populated countries such as China and India. The data that are available, however, allow us to presume that the situation in these countries with respect to anthrax is quite bad. According to data we obtained from the regional bureau of WHO for Southeast Asia (in New Delhi, India), in the state of West Bengal alone in 1979-1980 127 outbreaks of anthrax among cattle were reported; 831 animals were stricken with the disease, 479 of which died. One can assume that these cases must have been accompanied by illness among humans as well. In other Asian countries that submit information to WHO, a shortage of medical personnel means that only a small number of the actual cases of illness are reported.

According to WHO data (table 3), the highest number of cases of anthrax among humans is seen in countries with developed livestock breeding that lack an economic base and adequately developed veterinary services that are needed to organize mass vaccination of livestock. We are referring primarily to Iran, Iraq, and Turkey (although over the past 10 years the incidence of disease in Iraq and Turkey has declined significantly in comparison with previous decades). Isolated cases of illness in humans are reported in the majority of countries in the Middle East. Judging from data in the literature, a similar situation exists on the Indochinese peninsula and in Japan.

Table 3. The incidence of anthrax among humans in Asian countries between 1967 and 1977 (based on WHO data)

Страна / Country	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
Бирма (1)	.	.	.	.	.	.	156	.	.	.	.
Израиль (2)	—	1	—	1	—	1	—	—	—	—	—
Индонезия (3)	.	.	.	.	.	.	.	.	.	.	91
Иордания (4)	—	—	1	—	1	—	.	.	.	.	.
Ирак (5)	106	116	83	89	142	102	134	155	224	269	249
Иран (6)	1406	1677	1227	1397	949	721	586	681	579	.	.
Кувейт (7)	.	.	.	.	.	.	.	.	—	—	.
Ливан (8)	2	1	—	—	—	—	.	.	.	.	.
Саудовская Аравия (9)	.	.	.	.	.	.	.	.	17	6	1
Сирия (10)	4	6	.	—	3	7	9	4	2	7	.
Таиланд (11)	.	.	.	.	.	.	9	10	20	24	19
Турция (12)	1159	794	849	919	893	.	697	526	503	305	.
Филиппины (13)	14	63	24	.	38	13	33	.	.	.	.
Шри Ланка (14)	35	.	.	.	.	—	.	—	.	.	.
Япония (15)	4	1	3	2	1	3	3	—	—	—	.

1. Burma
2. Israel
3. Indonesia
4. Jordan
5. Iraq
6. Iran
7. Kuwait
8. Lebanon
9. Saudi Arabia
10. Syria
11. Thailand
12. Turkey
13. Philippines
14. Sri Lanka
15. Japan

The primary path of infection for humans in Asian countries is contact with animals infected with anthrax in the process of tending them and in cutting up carcasses. Cases of illness are reported primarily among livestock workers and members of their families [5].

In African countries (table 4) the highest number of cases of this disease among humans is reported in Upper Volta, Kenya, Ethiopia, Tanzania, Angola, Mali, and Uganda. In addition to those countries that report to WHO on the incidence of anthrax among humans, data in the literature offers evidence of the presence of the disease in Senegal, Morocco, Zimbabwe, and other countries [13, 14, 16, 17]. In light of this, we can assume that anthrax is much more widespread on the African continent than the official reports to WHO would indicate.

As a result of regular immunization of livestock, the incidence of the disease among animals as well as humans has declined significantly in a number of African countries. In Kenya, for example, between 1957 and 1966 there were 700-1000 cases of humans infected with the disease reported annually; in the last decade this number has not usually exceeded 200-300; a similar situation has developed in other African countries as well.

Table 4. The incidence of anthrax among humans in African countries between 1967 and 1977 (based on WHO data)

Страна Country	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
Ангола (1)	132	62	100	108	69	136	81	10	10	.	.
Бурунди (2)	.	.	.	.	.	20	.	.	.	.	.
Верхняя Вольта (3)	.	2	448	573	.	.	346	222	194	.	497
Гана (4)	.	.	10	132	22	.	18	3	17	28	10
Гвинея (5)	.	.	.	.	.	39	.	44	.	18	.
Египет (6)	.	.	.	.	.	.	135	.	.	—	.
Заир (7)	.	.	.	.	.	12	3	.	.	.	.
Замбия (8)	68	24	.	.	.	.	.	.	.	.	.
Кения (9)	258	380	307	169	225	229	282	.	.	325	.
Ливия (10)	31	.	18	.	.	.	37	19	17	.	.
Мадагаскар (11)	.	.	.	.	.	.	3	.	.	.	.
Мавритания (12)	.	.	.	.	.	.	.	58	.	21	10
Маврикий (13)	.	.	5	—	.	.	.	.	—	—	—
Малави (14)	.	.	.	.	20	.	.	.	.	.	.
Мали (15)	.	20	152	134	104	107	55	87	.	34	43
Нигерия (16)	2	2	97	2	9	73	.	48	65	.	.
Руанда (17)	26	32	62	24	22	12	19	—	12	62	166
Свазиленд (18)	.	.	.	.	.	.	.	.	1	3	.
Сенегал (19)	.	.	.	.	.	.	.	55	17	43	.
Судан (20)	.	80	174	112	.	10	742	.	101	.	.
Танзания (21)	672	128	.	221	.	.	.	.	.	.	.
Того (22)	.	.	.	.	.	2	.	.	.	.	.
Уганда (23)	24	16	16	98	850	.	135	281	70	.	—
Чад (24)	.	.	.	70	.	54	16	81	91	72	.
Эфиопия (25)	.	.	.	.	382	573	.	.	.	.	.
ЮАР (26)	68	24	12	1	.	.	.	.	.	.	.

Key:

1. Angola
2. Burundi
3. Upper Volta
4. Ghana
5. Guinea
6. Egypt
7. Zaire
8. Zambia
9. Kenya
10. Libya
11. Madagascar
12. Mauritania
13. Mauritius
14. Malawi
15. Mali
16. Nigeria
17. Rwanda
18. Swaziland
19. Senegal
20. Sudan
21. Tanzania
22. Togo
23. Uganda
24. Chad
25. Ethiopia
26. South Africa

In zones of pasture livestock breeding, there are often epizootics of anthrax among livestock and outbreaks of the disease among the nomadic herders. Humans in Africa are infected through direct contact with diseased animals, as well as in the process of skinning animals, cutting up carcasses, handling raw materials of animal origin and meat from diseased animals; cases of infection have also been seen that are the result of using brushes made of bristles and bites from blood-sucking arthropods [13].

The data presented by Sirol et al. [16] cast some doubt in this regard; according to these findings, in the majority of 22 cases of humans infected with anthrax observed by the authors in Upper Volta in 1972, using meat from

diseased animals in food products was cited as the reason for the infection. Apparently, it is more likely that the infection occurred not as a result of using the meat as food, but as a result of cutting up the carcasses. Evidence of this can be found in the data on localization of anthrax carbuncles on various exposed parts of the body.

In Australia and Oceania there are very few reported cases of humans infected with anthrax: 1-3 cases per year (only in 1972 in Australia were 11 cases of this disease in humans reported). This disease among humans is tied primarily to contact with cattle or with wool from infected sheep. The disease in humans and animals has been observed only in two states: New South Wales and Victoria, and it is concentrated between 34° and 36° south latitude and 14 [as published] and 148 east longitude. In New Zealand, Papua, and New Guinea, separate cases of the disease among humans have been seen, but the actual number is unknown.

Thus, it is possible to say that today the incidence of anthrax among humans is significant, primarily in the developing countries of Asia, Africa, and Latin America. Immunization of livestock will help reduce the incidence of the disease among animals and humans, but it will not lead to its elimination.

The epidemiology of anthrax in developing countries has certain traditional characteristics which indicate that humans are infected mainly through tending and slaughtering animals and when cutting up the carcasses; while the epidemiological peculiarity of anthrax in developed countries is the predominance of occupational morbidity, that is, the result of infection from contaminated raw materials of animal origin, and mainly those that have been imported.

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MOLECULAR ORGANIZATION AND FUNCTIONS OF PROTEIN TOXINS THAT ARE CAUSATIVE AGENTS OF CHOLERA AND PLAGUE

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 (manuscript received 24 Mar 83) pp 7-13

[Article by V. I. Yefremenko, Volgograd Antiplague Scientific Research Institute]

[Text] Research on bacterial toxins began at the end of the 19th century with the isolation of the diphtheria toxin, and other bacterial toxins were isolated and studied later. It was established that the majority of bacterial toxins have a protein nature and have receptor and activator functions. The part of the toxin molecule that fulfills the receptor function makes it possible for the toxin to attach itself to certain cellular receptors, and then the activator part carries out the toxic function of the preparation. Research results have shown that a toxin lacking the receptor part of the molecule is practically incapable of having any biological effect, in spite of the fact that it still has the section of the molecule that performs the activating function [2].

As more information has been gathered on bacterial toxins, the need has arisen to systematize the data that has been obtained and to create a classification system. The basic, well-known classifications of bacterial toxins have been described in a number of monographs and reviews [1, 2] and Bonventre [11] proposed a classification of toxins based on the structural peculiarities of the toxic molecules. According to this classification all bacterial toxins are divided into 4 groups: 1) simple toxins that at the stage of the precursor toxins have one polypeptide chain, the activation of which follows the proteolysis model; during activation they become true toxins and perform receptor and activator functions; 2) complex toxins that have two or more polypeptide chains and are synthesized separately from each other (this group includes the protein toxins that are causative agents of cholera and the plague); 3) toxin mixtures that consist of several unconnected components; and 4) conjugated toxins that are active when combined with a prosthetic group. Results of research on bacterial toxins from the position of the interconnection between their structure and function have cast doubt on the accuracy of isolating the third and fourth groups in Bonventre's classification.

After establishing the leading role of the cholera toxin (cholerogen) in the pathogenesis of cholera, it was determined that the cholerogen is a thermolabile protein with a molecular weight of 84,000 atomic units and consisting of 2 fragments: fragment A performs the activator function and

fragment B, or the cholerogenoid, performs the receptor function. Both fragments consist of subunits [2, 9, 18, 45, 53].

The fact that the cholerogen molecule consists of two fragments, A and B, that are distinct in terms of their structure and properties suggests the involvement of several genes in the synthesis of the cholera toxin [7]. This supposition was confirmed when vibrions were obtained that are involved primarily in the sythesis of one of the fragments [18, 22, 23, 42]. When animals were immunized with toxin fragments A and B, an approximately equal level of defense was obtained against subsequent introduction of the cholerogen [34, 49], even though immunoglobulins for fragments A and B are serologically distinct [46].

According to data found in the majority of research studies, fragment A of the cholerogen has a molecular weight of 2800 atomic units and it consists of 2 subunits that are bound to each other by a disulfide bond. Under the influence of chemical substances that reduce the S-S group to free S-H groups (dithiotreitol, 2-mercaptoethanol), fragment A splits into 2 subunits, which can be rejoined during oxidation to form fragment A. The results of studies to determine the molecular weight of the subunits of fragment A conducted by a number of researchers were not identical. Today the molecular weight of the subunit of fragment A that is directly involved in the biological activity of the cholerogen is recognized to be about 23,000 atomic units, and the subunit that forms the bond between fragments A and B of the cholerogen is thought to be 5000 atomic units [15, 17, 18, 28] [m. w. s. as in text]

Fragment B, or the cholerogenoid, has a molecular weight of 56,000 and it consists of identical subunits that include 103 amino acid radicals; this suggests that fragment B contains 5 subunits with a molecular weight of no less than 12,000. The noncovalent bond between the separate subunits can be broken when fragment B is reduced or alkylated, when it is exposed to sodium dodecylsulfate, 6 M of urea with an acidic pH, or 6 M of guanidine hydrochloride [2, 15, 18, 28, 53].

Data that have been obtained on the amino acid composition and the amino acid sequence of the cholerogen and its subunits have demonstrated the absence of hydrophobic surfaces in these compounds [55]. Trypsin can split the subunit of fragment A with a molecular weight of 23,000 [29], which in the cholerogen molecule is shielded by other structural components with a high proline content, which forms a bond with lysine and arginine [41]; this provides the toxin molecule with a high degree of resistance against the effects of proteolytic enzymes. In the study of the detoxifying effect on the cholerogen exerted by various aldehydes, it was also established that sections of the molecule that perform the toxic functions are located mainly inside the protein globule and are subject to the effect of a high molecular aldehyde (benzaldehyde) only after the protein globule is destroyed in the presence of urea [3]. When the cholerogen is exposed to the combined effect of dithiotreptol and 2-mercaptoethanol, it breaks down into separate subunits with a loss in its original toxicity [18, 53]. The toxic function of the cholerogen is inhibited by chemical compounds that react specifically with arginine and NH_2 groups, whose modification removes the toxin's receptor function [3, 32,

33]. The cholerogen's toxicity decreases while its immunogenicity and antigenicity are preserved also when it is exposed to gamma radiation [6].

Results from studies on the mechanism of action of the cholerogen on a macroorganism have shown that the toxin interacts with monosialogangliosides G_{MI} that are part of the structure of eucaryotic cell membranes in a molar ratio of 1:1; both fragment B of the cholerogen and its separate subunits are capable of bonding with the ganglioside [20]. It has been established that the toxin attaches to the oligosaccharide part of the ganglioside molecule [30, 52], using the part (subunit) of fragment B that contains arginine. This same part of the toxin molecule is responsible for the interaction with the antitoxic immunoglobulin [16]. These data explain the loss of the ability of the cholerogen or cholerogenoid, having been acted upon by the antitoxic immunoglobulin, to fix onto the ganglioside G_{MI} [2, 15, 53]. One molecule of cholerogen bonds with several molecules of the G_{MI} gangliosides [19], the quantity of which represents less than 5 percent of the total number of gangliosides in the cell [47]. After the toxin has formed complexes with the G_{MI} gangliosides, the complexes migrate in the plane of the membrane to the poles of the cell [24], there is reduction of the disulfide bridge that joins fragments A and B, and fragment A passes through the membrane into the cell [51].

In recent years a great deal of attention has been given to studying the role of neuraminidase in carrying out toxins' biological functions. With cholera, it alters various gangliosides to the structure of G_{MI} [2], which provides an increase in the number of toxin receptors in the intestine (this also probably applies to other infections in which toxins are produced that interact with monosialogangliosides). With illnesses caused by agents of tetanus, botulism, and the like, the toxins of which interact with di- and trisialogangliosides [2], the neuraminidase also probably transforms the gangliosides to the G_{MI} structure. Here, in our opinion, the toxin is not capable of fixing itself to the surrounding cells and tissues of the macroorganism and it penetrates beyond the limits of localized microbes, infecting, for example, the cells of the primary tissue and so forth. This supposition is confirmed indirectly by data that show the botulin toxin is not absorbed in the intestine, but ends up in the lymph [44].

When fragment A penetrates into an intestinal cell, it catalyzes the hydrolysis of NAD to nicotinamide and ADP-ribose, after which adenyl cyclase is activated and catalyzes the production of cyclic 3'-5' adenosine monophosphate (cAMP). As the result of an as yet unknown mechanism, this promotes secretory transport of Cl^- and HCO_3^- ions through openings in the intestine; and as a result of osmotic shifts, sodium ions and water also pass through and the intestinal nervous system is activated. One or several molecules of cholerogen are sufficient to activate cAMP in eucaryotic cells during a period of contact lasting several minutes and the action of the toxin lasts for the entire life of the cell. The accumulation of cAMP in the cell begins after 60-90 minutes, and reaches its maximum after 3 hours [9, 12, 18, 21, 43, 48, 53].

Numerous observations have been described regarding the effect of a cholerogen on various systems, organs, tissues, and cells of a macroorganism. For example, a cholerogen in the intestine stimulates the secretion of water and

electrolytes (that is, it performs an enterotoxic function); it causes a disruption in capillary permeability when it is introduced into the skin (the skin permeability factor); it modifies various proteins; it inhibits the development of delayed hypersensitivity; it stimulates steroidogenesis in a culture of adrenal cells; it increases lipolytic activity in fat cells; it causes abortions and promotes the development of sterility in female mice; it affects the rhythm of heart contractions; it leads to a change in the morphology of cultured adrenal cells or Chinese hamster ova; it affects the reduction of bone tissue; it releases growth hormone from the hypophysis; it potentiates the release of insulin from the pancreas; it intensifies the secretion of glycoprotein in the intestine; it suppresses blast transformation of lymphocytes; it reduces the number of cells that form antibodies; it inhibits chemotaxis of polymorphonuclear leucocytes; and so on [2, 9, 14, 18, 19, 21, 50, 53]. The cholerogen's broad range of action can be explained not so much by the presence in its structure of molecules with different centers that activate various cells of the macroorganism, but by the universal nature of its effect on these cells, the functions of which are extremely varied. Consequently, their reactions to the cholerogen are diverse; in a number of cases the action of the cholerogen is compared to hormonal action [54]. The varying sensitivity of different cells and tissues to the cholerogen [31] and the minor conformational changes in the toxin molecule that arise in the process of its secretion, and the purification and action of various chemical compounds can, in our opinion, reduce the toxin's tropistic relationship to various cells, and thus the cholerogen's functions (enterotoxic, cutaneous, etc.) can either be reduced or disappear altogether in various biological reactions. For example, when the cholerogen is exposed to ammonium sulfate, its enterotoxic function disappears almost entirely, while it retains the ability to cause destruction of capillary permeability with intracutaneous introduction. Similar results were obtained with nonenzymatic carbamylation of a cholerogen [3]. Thus, comprehensive research on the cholera vibrion toxin has made it possible to determine a number of the aspects of the relationship between its structure and biological functions.

Protein thermolabile plague toxins have been studied to a much lesser extent. After Yersen discovered the causative agent of plague, numerous efforts were made to isolate the substance in the bacteria that is responsible for the intoxication of the organism. Relatively little is known today about the mechanisms of action of Yersinia pestis on the macroorganism, although the large volume of data in the literature involving observations of people stricken with plague has provided a basis for supposing that they have pronounced toxemia, especially in those cases when the illness was extremely sudden and those dying from plague had the typical symptoms of so-called toxic death [25]. A protein that is highly toxic to mice and rats has been discovered in the plague agents and it is called "mouse" toxin. Protein fragments were also isolated, which by themselves even in large concentrations did not kill guinea pigs; but when mixed together these fragments were toxic to these animals in relatively small doses, which indicates their synergistic effect [13]. A plague microbe protein toxin has been described that causes death in mice and guinea pigs [8]. This review summarizes material that is devoted to the study of protein toxins that are toxic to white mice. In 1964 Montie et al. [38] isolated 2 toxins (A and B) from the plague microbe that differed in terms of their physical and chemical properties, one of which was identical to

the "mouse" toxin described earlier. The LD_{50} for mice with intraperitoneal introduction of these preparations was 0.5-1.0 micrograms of protein [37]. Results of research on the protein toxins A and B have shown that mice and rats are highly sensitive to these toxins, while other animals are resistant to their effects [5, 25]. Toxin A, attached to the membrane of the plague agent, has a molecular weight of 240,000 and its electrophoretic mobility in a polyacrylamide gel is less than that of toxin B. Toxin B, which exhibits more mobility during electrophoresis is found in the cytoplasm of the agent and has a molecular weight of 120,000. Toxins A and B form individual zones of precipitation with antitoxic sera in immunochemical reactions [38]. Toxin A is a type-specific antigen for the plague agent and pseudotuberculosis [4]. Toxins A and B have practically identical amino acid compositions, but it has been determined that there is 33 percent less tryptophan in toxin B than in toxin A [39].

When toxins are treated with detergents they dissociate into subunits with a molecular weight of 24,000 that contain 2 polypeptide chains with a molecular weight of 12,000, one of which is found in both toxins A and B. In light of this, it is supposed that toxin A consists of 10 subunits and toxin B of 5. The subunits retain a toxicity that is equivalent to that of the original preparations. Approximately 60 percent of the polypeptide chains of subunits of toxins A and B have the same electrophoretic mobility during electrophoresis in a polyacrylamide gel; the remaining 40 percent differ in terms of their electrophoretic mobility and the polypeptide chains of subunits of toxin B are more mobile [37, 40]. Assuming that toxin A is a dimer of B toxins, an effort was made to obtain toxin B from toxin A. However, the incubation of toxin A in solutions with various pH levels and 2-mercaptoethanol that destroy the disulfide bonds, did not lead to the dissociation of toxin A [39]. When animals were immunized with immunochemically pure toxins A and B, both homologous antibodies and antibodies for another toxin were discovered in the serum of the immunized animals. After the immunochemically homogeneous solution of toxin A was stored at 37°C for 2 months, toxin B appeared in serological reactions in the preparation. These results confirmed the presence of several common structural peculiarities in toxins A and B [4].

The heating of the purified "mouse" toxin performed by Ajl et al. [10] and the effect of ultrasound and formalin on the toxin led to a decrease in or complete disappearance of the preparation's toxicity. Proteolytic enzymes (trypsin, L-chymotrypsin, papain) did not have any effect on the serological and toxic properties of the purified toxin after 1-7 days of incubation at 37°C, in spite of the appearance of free amino acids that was observed. Differences were discovered in the tertiary structures and in the sequential arrangement of the amino acids in toxins A and B [39]. When the subunits of toxins A and B were studied, a difference was discovered in the tryptophan content of the polypeptide chains [40]. Toxin A turned out to be more sensitive to urea, desoxycholate, digitonin, 5-fluorotryptophan, and lyophilization [38, 39]. Using a chemical modification method, it was determined that the active sections of the polypeptide chains that are responsible for carrying out the toxic funcion contain amino acids with free NH_2 groups. Destruction of the disulfide bonds through oxidation or reduction caused no changes in the toxicity of the plague toxins [4]. These results and the data obtained by Montie et al. [40] showing that relatively short polypeptide chains (subunits)

of toxins A and B perform the toxic function, allow us to assume that the disulfide bonds have practically no effect on the performance of these functions by protein toxins that are plague agents. It has been established that the effect of 6 M urea on toxic fragments of plague agents was an increase in the preparation's toxicity due to individual destruction of the polypeptide chains of toxin molecules and the exposure of new functional groups of molecules that perform the toxic functions. Treating the preparations with aldehydes of varying molecular weight showed that the groupings of toxin molecules responsible for the toxic function are essentially shielded and a high molecular aldehyde can have an effect on them only after there is a change in the spatial structure of the toxins that is the result of 6 M urea [4]. The results of studying the mechanism of interaction between plague protein toxins and mitochondria from bull and rat hearts showed that with a low concentration of hydrogen ions (pH 11.0), the "mouse" toxin interacted with the structural protein of the mitochondria, forming a complex primarily with toxin A. An almost complete joining of the toxin with the structural protein of the mitochondria took place when the ratio between the protein and toxin was 20:1; one molecule of the toxin bonded with several molecules of the mitochondrial structural protein [25-27, 36]. On the basis of these data one can assume that the mitochondrial structural protein is the receptor for the "mouse" toxin and the toxic function of the toxin works mainly at the level of the exchange processes in the mitochondria of animals that are susceptible to the toxin.

Quite a large body of research has been devoted to the study of the mechanism by which plague toxins affect the macroorganism and the exchange of substances in animals susceptible to this toxin. In the majority of cases, however, unpurified preparations were used as toxins. Direct research on the effect of "mouse" toxin became possible only after the development of methods for purifying the toxin. It was demonstrated that when introduced into mice, the purified toxin caused bristling of the hair, inertia, a rise in body temperature, difficulty breathing, a drop in blood pressure, and shock. The mice developed leucopenia, eosinopenia, expansion of blood vessels, and thickening of the blood with an increase in erythrocytes up to 35 percent. Subcutaneous or intracutaneous introduction of the toxin at first caused formation of edema at the site of application, and later necrosis of the tissues. At the site of the toxin's application, there was an increase in the permeability of subcutaneous capillaries. A histological examination of mice that died during the first 10 hours following introduction of the toxin showed swelling of the endothelium in pulmonary vessels, hyperemia in organs, and fatty degeneration in the heart muscle. Animals that died later on showed hyperemia of the spleen, degeneration of the endothelium in kidney tubules, and fatty infiltration in the liver [25]. It was also determined that the "mouse" toxin inhibits the oxidation of alpha-ketoacids in homogenates in the mouse liver and spleen, as well as in acellular extracts secreted by Escherichia coli; the toxin had little effect on the oxidation of succinic acid and citric acid. The toxin suppressed the exogenous respiration of mitochondria isolated from the hearts of mice and rats, but not those of rabbits, dogs, and monkeys, which are not very sensitive to the toxin's effects. A connection was discovered between the toxicity of a toxin molecule in vivo and its ability to inhibit mitochondrial respiration [26, 36]. When the permeability of the mitochondrial membranes of a rabbit's heart was destroyed by desoxycholate or ultrasound, the "mouse" toxin inhibited the respiration of these mitochondria.

The toxin's effect was seen in the transport of electrons along metabolic pathways between nicotinamide adenyldinucleotide phosphate (NADP) or a salt of succinic acid and cytochrome B by retarding the enzymatic activity of dehydrogenase. It was discovered that the toxin inhibited the accumulation of calcium and inorganic phosphate ions in the mitochondria. When the toxin was inactivated by formalin, the inhibitory action disappeared. When the toxin interacted with the mitochondria, the mitochondria swelled up; the toxin caused swelling only in those mitochondria in which it had inhibited exogenous respiration. In the presence of cyanides and sodium azide, no mitochondrial swelling was observed and the toxin did not have any effect on mitochondria from the brains of mice and mitochondria from animals immunized with the toxin [25]. In the opinion of Montie [35], "mouse" toxin acts as a beta-adrenergic blocking agent in the macroorganism.

The data presented here offer evidence of the need to study biologically active molecules taking into consideration the close connections between their structural peculiarities and various functions, which opens up new possibilities in understanding the mechanisms of their action on the macroorganism and points the way for developing various preventive and therapeutic preparations.

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PARTIAL ANTIGENIC STRUCTURE OF GROUP H10 FLAGELLINS IN ESCHERICHIA COLI AND ITS GENETIC DETERMINATION

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[Text] Enterobacterial flagella, including E. coli, consist of a protein-flagellin that is the sole component of the flagellum filament. Therefore, the antigenic specificity of the flagella (the H-antigen) is determined by the corresponding properties of this protein, the primary structure of which is coded in E. coli by the hag gene that is located on the 42nd minute of the genetic chart of E. coli K12 [5], and possibly in several strains, by another structural gene ith a different location. Different alleles of the hag gene determine the synthesis of various flagellins that account for the corresponding different antigenic and morphological properties of the flagella [8]. Salmonellae were used as an example to demonstrate the possibility of natural modification of a synthesized flagellin before it is formed into the flagellin structure [16]. The H-antigen is an important differential diagnostic sign for identification of bacteria.

A total of 53 varieties of H-antigen have been officially recorded for E. coli (H1-H12, H14-H21, H23-H49, H51-H56), which represent the order in which they were recorded [14]. Their partial structure has not been adequately studied, although it is known that some H-antigens designated by the same numbers are not identical and differ in terms of their partial structure [3, 4, 9-11]. Utilizing this information would provide an additional opportunity for more reliable differentiation of the bacteria and for establishing their etiological and epidemiological role in infectious diseases.

Among the antigens designated as H10, 4 variants have been discovered; 3 of them were found in Escherichia 055:K59 [4]. It is still not known whether these variants are found among Escherichia of other O (OK)-groups: whether there are other variants of antigens in the H10 group; and finally, whether these differences in the partial structure are accompanied by differences in the corresponding flagellin-coding genes or if they are the consequence of various modifications in the originally identical flagellin. The present article deals with these questions.

Materials and methods. The following strains of E. coli were used in the present study: strains with a previously identified partial structure of the H-antigen [4], including the H-test strain Bi623-42 with the serotype O11:K10:H10 (a,b,c,d); B581 strains with the serotype O55:K59:H10 (a,c,e,f,g), 552-O55:K59:H10 (a,b,c,e,g,h) and c97/53-O55:K59:H10 (a,b,g,i); the O- and K-test strains of the International Collection of Escherichia A12 b (O6:K54:H10), ES1483 (O149:K91, 88a, 88c:H10), 880-67 (O151:K-:H10); strains with the serotype O5:K*:H10 that we obtained from healthy people; strains with serotypes O151:K-:H10, O19:K*:H10, and O8:K84:H10 that were sent to the All-Union Escherichia Center for identification of the H-antigen; strains obtained during acute intestinal illnesses; and R-form strains (OR:H10) that were obtained from individuals with pyelonephritis. In experiments involving the transduction of the H-antigen the B99-2 strain with flagellum antigen H6 was used as the recipient, in addition to the variant of flagella produced by it spontaneously that has an altered antigenic specificity [15]. The entire set of H-test strains from the International Collection of Escherichia was used, in addition to strains whose H-antigens are distinguished from identical types [3, 4]. Transduction was performed using a thermoinducible phage P1c1r100Km^{3} [15].

For the serological research we used H-sera for all the type H-antigens and for all the strains being studied with the H10 antigen, which were prepared according to the standard method [1]. The sera for the partial factors of the group H10 antigens were prepared on the basis of the previously determined partial structure according to the system presented in table 1. Table 1 also indicates the dilutions (expressed in terms of the native serum) used in the agglutination assay reaction to indicate the corresponding partial factors.

The methods used for the H-agglutination assay reaction (the starting dilution of the serum with an antibody titration of 1:25), for the adsorptions of H-agglutinins, and the transduction of H-antigen specificity corresponded to the methods described previously [1, 15]. With a relatively low phage titer or with transduction that was not sufficiently effective, we used the method developed by Yu. A. Ratiner for accumulating transductants: we mixed 1-4 ml of fluid culture containing the recipient with 1-5 ml (in an equal or larger amount) of the phage and after 30 minutes of sorption at 30°C the mixture was transferred to a flask (100 ml) with 50-60 ml of Hottinger's culture containing a serum (1:100) for the H-antigen of the recipient and 2-2.5 percent glycerine; this was incubated at 30°C for 2-3 days and the layer above the sedimentation was placed in the center of a selective medium (a semi-fluid medium with a serum for the H-antigen of the recipient) in a Petri dish. For counterselection of the B99-2 recipient we used simultaneously the H6 serum and the adsorbed serum for the H-antigen of the spontaneous variant of this strain. The addition of glycerine helped maintain a high level of flagella in the bacteria over the long culture period.

In separate transduction experiments we introduced into the semi-fluid medium an additional adsorbed serum to the specific H-antigen factor of the donor in dilution, which (in the control) immobilized the donor cells. The transductants were cultivated at 30°C.

In all the transduction experiments for a control we used a recipient culture that had not been treated with phage, but was cultured under the same conditions as the transduction mixture.

Table 1. The system used to obtain sera for the partial factors

Парциальные факторы (1)	Нативная сыворотка к штамму (2)	Штамм-адсорбент (3)	Титр антител к парциальному фактору (4)	Использовавшееся в реакции агглютинации разведение (5)
c, d	Bi 623-42	c 97/53	c 3200	1:100
g, i	c97 53	Bi 623-42	i 6400	
			g 800	1:100
i	c97 53	552	i 3200	1:100
h	552	Bi 623-42 +B581	h 800	1:100
d	Bi 623-42	552	d 50	1:10
e, f	B581	Bi623-42 +c97/53	e 400	
			f 100	1:25
f	B581	552	f 100	1:25
b, h	552	B581	b 1600	
			h 400	1:50
e, g, h	552	Bi623-42	e 25, g 100, h 1600	1:50

Note: Here and in table 3 the antibody titers are represented by inverse indicators.

Key:

1. Partial factors
2. Native serum for the strain
3. Strain-adsorbent
4. Titer of antibodies for the partial factor
5. Dilution used in agglutination reaction

Results and discussion. Table 2 illustrates an analysis of the group H10 antigens using factor sera; it is evident from this table that the H-antigens of strains of the serological groups 06:K54 and 08:K84 behaved similarly to the standard H10 (a,b,c,d) antigen of the Bi623-42 strain; and the H-antigens of the strains of the serological groups 05:K^{-} and 0149:K91,88 (a,c) behaved similarly to the H-antigen of the c97/53 strain, that is, H10 (a,b,g,i). The H-antigens of the strains of the serological groups 019:K^{-} and 0151:K- had no analogues. Flagellar antigens of strains with the O-antigen in the R-form differed from the standard; and as demonstrated by the results of tests with sera cd, gi, i, and h, there was a similarity with the H-antigen of the B581 strain (3 strains) and the c97/53 strain (4 strains).

Experiments on cross-adsorption of agglutinins from sera prepared for the strains being studied confirmed that the H-antigen of the 1420 strain and the standard H-antigen are absolutely identical and that the H-antigens from Escherichia 05 and 0149 and the flagellar antigen of the c97/53 strain are absolutely identical. The H-antigen of the A12b strain differed from the standard H10 antigen in that it contained only a part of the d(d^{1}) factor and an additional, but weak (residual antibody titer of 1:50) specific antigen factor that is absent in all other strains. We used the symbol k to denote

this factor and we used d^1 and d^2 to denote the two components of factor d of the standard H-antigen.

Table 2. An analysis of group H10 flagellar antigens using factor sera

Серологическая группа E. coli (1)	Штамм или количество штаммов (2)	(3) Результаты взаимодействия с факторными сыворотками								
		cd	gi	i	h	d	ef	f	bh	egh
O11 : K10	Bi623-42	+	−	−	−	+	−	−	+	−
O65 : K5	B581	+	+	−	−	−	+	+	−	+
O55 : K59	552	+	+	−	+	−	+	−	+	+
O55 : K59	c97/53	−	+	+	−	−	−	−	+	+
O8 : K84	1420	+	−	−	−	+	−	−	+	−
O6 : K54	A12b	+	−	−	−	+	−	−	+	−
O149 : K91.88(a, c)	ES1483	−	+	+	−	−	−	−	+	+
O6 : K·	8 штаммов (4)	−	+	+	−	−	−	−	+	+
O151 : K−	5 »	+	+	−	−	−	+	−	+	+
O19 : K·	3 »	−	+	+	−	−	−	−	+	−
OR	4 »	−	+	+	−	.	.	.	.	.
N	3 »	+	+	−	−	.	.	.	.	.

Key:

1. Serological group of E. coli
2. Strain or number of strains
3. Results of interaction with factor sera
4. Strains

As demonstrated by the results of cross adsorption of antibodies, the flagellar antigens of strains 0151:K- and 019:K· were identical within the limits of each of these serological groups. They were studied further using the 2599 and 880-67 (0151:K-) and 30T (019:K·) strains as an example. After the adsorption of the sera 2599, 880-67 of the 552 strains and serum 30T by the c97/53 strain, antibodies remained (1:400, 1:800, and 1:3200, respectively) that were specific only to H-antigens of 0151 and 109 Escherichia, respectively; that is, each of these 2 types of H-antigens had a specific factor. We used the symbols l(y 0151) and m(y 019) to denote these factors. Furthermore, these results showed that the remaining partial factors of these H-antigens are represented entirely in the H-antigens of the adsorbent strains. Proceeding from the known partial structure of the H-antigens of strains 552 and c97/53 and the data in table 2, one can conclude that the 0151 Escherichia had factors b, c, g, e, and possibly a, but not factor f; and that the 30T strain had factors b, i, and possibly a, but not factor g.

The results of the experiments presented in table 3 illustrate the extent to which these factors correspond to those that were determined earlier. Both variants of the H-antigen contained factor b entirely, since the adsorption of serum bh by either variant led to a complete removal of the corresponding antibodies. Factor c, which had been determined earlier, consisted of two components--c^1 and c^2, since the 2599 strain contained only part of it (c^1). Two components were also found in factor a--a^1 and a^2, one of which (a^1) was found in the H-antigen of the 2599 strain, and the other (a^2) was found in the H-antigen of the 30T strain. This follows from the results of the adsorption of the serum for the H-antigen of strain c97/53 (see table 3): the preservation of antibodies for the standard H-antigen after adsorption of this serum by the 2599 or 30T strain can be caused only by incomplete adsorption of

antibodies for factor a. The disappearance of these antibodies after total adsorption by strains 2599 and 30T is evidence that these strains contained different components of factor a. Factor i also turned out to have two components (i^1 and i^2), and the 30T strain contained only factor i^1. The results of studying the 880-67 strain were analogous to the data presented in table 3 that were obtained for the 2599 strain. Besides the antigenic partial factors studied above, the H-antigens of the 30T strains and the O151 serological groups had one more common component for which an antibody remained after adsorption of the serum for the 30T strain by the 552 strain separately or in combination with B581. However, these antibodies were not found in a study of the sera for 4 strains of the O151 serological group.

Table 3. An analysis of partial factors a, b, c, i

(1) Анализируемый антигенный фактор	(2) Адсорбированная сыворотка	(3) Штамм для дополнительной адсорбции	(4) Титр Н-антител к штамму					
			B1623-42	B581	552	c97/53	2599	30T
b	bh	—	1600	—	1600	1600	1600	1600
		2599	—	—	400	—	—	—
		30T	—	—	400	—	—	—
c	cd	—	3200	3200	3200	—	1600	—
		2599	800	400	800	—	—	—
a	сыворотка c97/53, адсорбированная штаммом 30T (5)	—	800	800	800	1600	800	—
		2599	—	—	—	200	—	—
	сыворотка c97/53, адсорбированная штаммом 2599 (6)	—	800	800	800	6400	—	800
		30T	—	—	—	20	—	—
i	i	—	—	—	—	3200	—	3200
		30T	—	—	—	200	—	—

Key:

1. Antigenic factor being analyzed
2. Adsorbed serum
3. Strain for additional adsorption
4. Titer of antibodies for the strain
5. Serum c97/53, adsorbed by strain 30T
6. Serum c97/53, adsorbed by strain 2599

Due to differences in the partial structure, strains with different variants of the H-antigen agglutinated in different ways in the (native) sera obtained for them. Sera c97/52, 552, and 2599 agglutinated all the strains to approximately identical dilutions. The 30T serum agglutinated strains 30T and c97/53 to the titer, and the rest of the strains to one-fourth the titer. Of the 3 sera for the standard H-antigen, only 1 agglutinated strains 2599, 880-67, and 30T to half the titer and the other 2 agglutinated them to just one-eighth the titer.

It is possible that this was the reason for the originally erroneous identification of the H-antigen of the 880-67 strain as a new antigen [12, 13]. Two-way connections with other H-antigens of Escherichia were found only between the H-antigen of strains in the serological group 0151 and the standard H42 antigen.

Table 4 contains the results of transduction of the H-antigen from a strain with a different partial structure. Transductants were found only in the absence of antibodies for the specific factor of the donor antigen in the selective medium. Three transductant clones were selected from each cross to study the H-antigen. Adsorption of the sera for the donor strain by the transductants led to complete removal of the H-antibodies. This proves the correspondence between the H-antigen of the transductants and the H-antigen of the donor strain.

Table 4. Results of transduction of flagellar antigens of the H10 group

(1) Штамм-донор	(2) Присутствие в селективной среде антител к специфическому H-антигенному фактору донора	(3) Наличие диффузного роста трансдуктантов на селективной среде	(4) Тип антигена H10 у трансдуктантов
Bi623—42	—	+	a^1, a^2, b, c^1, c^2, d^1, d^2
B581	—	+	a^1, a^2, c^1, c^2, e, f, g
552	—	+	a^1, a^2, b, c^1, c^2, e, g, h
	+	—	
c97/53	—	+	a^1, a^2, b, g, i^1, i^2
	+	—	
880—67	—	+	a^1, b, c^1, e, g, l
	+	—	
30T	—	+	a^2, b, i^1, m
	+	—	

Key:

1. Donor strain
2. Presence of antibodies for the specific H-antigen factor of the donor in the selective medium
3. Diffusion increase in transductants in the selective medium
4. Type of H10 antigen in the transductants

This research made it possible to discover the existence of 7 variants of group H10 flagellar antigens in natural strains of E. coli with the following partial structure: a_1^1, a_2^1, b, c^1, c^2, d^1, d^2 (serological groups O11:K10 and O8:K84); a^1, a^2, b, c^1, c^2, d^1, k (serological group O6:K54); a^1, a^2, c^1, c^2, e, f, g_1 (O55:K59, strain B581); a^1, a^2, b, c^1, c^2, e, g, h (O55:K59, strain 552); a_1^2, a_2^2, b, g, i^1, i^2 (O5:K$^-$, O149:K91, 88a, 88c, and O55:K59, strain c97/53); a^1, b, c^1, e, g, l (O151:K-); and a^2, b, i^1, m (O19:K). The absence of freely migrating transductant clones in those cases when the selective medium contained an antigen for the specific factor of the donor H-antigen gives us

reason to believe that the expression of the donor specific factor in the transductants was not tied to cotransduction of any other genes, except for the hag gene, which would modify the product of the hag gene. This in turn allows us to believe that the partial structure of the H-antigens depends exclusively on primary structure of the flagellins that is coded in the corresponding hag alleles.

We cannot ignore the fact that all these variants of antigens in the H10 group arose at some stage in the evolution of E. coli as a result of mutations in some original allele in the hag gene. For example, it is known that a fairly broad spectrum of variants of antigen i in salmonella can be obtained as a result of mutations just in the H1(i) gene [6, 7]. A similar phenomenon was demonstrated in a number of H-antigens of E. coli [2]. But since identical variants of group H10 antigens are found in different OK-groups of Escherichia, they should be considered a stablized and characteristic indication of certain serotypes. Identification of variants of group H10 antigens using factor-specific sera would certainly be useful (for epidemiological and diagnostic purposes) in differentiating strain within serotypes; this is clear from the example of Escherichia 055:K59:H10 or R-form strains. Bacteriologists are encountering these strains more and more frequently in connection with the growing interest in the etiology of, for example, urinary tract diseases.

Conclusions

1. Flagellar antigens in Escherichia, identified as H10, are not identical and 7 variants have been determined among them that differ in terms of their fine partial structure.

2. Differences among variants of flagellins in the H10 group are the result of differences in the genetic information that is coded by the corresponding alleles of the hag gene.

3. Variants of the same type of group H10 flagellar antigens can be found in Escherichia of different O-groups, and different variants of group H10 variants can be found in Escherichia that are identical in terms of their somatic antigen complex.

4. Identification of variants of H10 group antigens using factor-specific sera should be useful for detailed differentiation of Escherichia with the aim of understanding the epidemiology and etiology of the diseases caused by them.

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LETHAL PROCESSES IN ESCHERICHIA COLI OCCURRING DUE TO THEIR DEHYDRATION IN AIR AT DIFFERENT HUMIDITY

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[Article by V. F. Konyukhov, L. A. Mal'tseva, and M. E. Lyayman, Epidemiology and Microbiology Institute imeni N. F. Gamaleya of the USSR Academy of Medical Sciences, Moscow]

[Text] Today it is known that humidity in the air has a substantial effect on the survival of viruses and bacteria in the air. There are varying data on the extent and nature of this effect, but the majority of authors note a heightened death rate among bacteria in the air in an average humidity range (50-70 percent) [4, 5, 9].

While studying the effect of dehydration on the viability of bacteria, we established that the reason for the death of the aerosolized microbial cells is the irreversibility of aggregation and conformational changes in the macromolecules as a result of rapid dehydration [1]. The degree of irreversibility of this aggregation and the conformational changes is, apparently, a function of the speed and degree of removal of free water and hydration water. The aim of the present work was to use biophysical methods to prove the suppositions regarding the reasons for the death of aerosolized bacterial cells at varying air humidity.

Materials and methods. We used the Wp-2 strain of E. coli B. In determining the amount of hydration water in the cells, we grew the strain in Hottinger's medium (aminic nitrogen 240 mg percent with 0.1 percent glucose added); when measuring the activity of beta-galactosidase, we grew the strain in a minimal medium 1-A [8] (with 20 mg/ml tryptophan and 0.4 percent lactose added). We incubated the culture for 18 hours at 37°C.

We used the method described earlier [2] to aerosolize and determine the viability of the microorganisms in a simulated aerosol state at three different levels of relative humidity--30, 50, and 80 percent.

We used the method described in our previous work to measure the characteristics of proton magnetic resonance.

The amount of hydration water in sample cells was measured by means of the nuclear magnetic resonance, using the method developed by Kuntz and Kauzmann [7] and the method developed by Daszkiewicz et al.

We measured the dry mass of bacteria by weighing samples that were moist and samples that had been dried in a drying chamber at 104°C.

The activity of beta-galactosidase in E. coli was determined in the original suspension and in cells exposed for 15 minutes in a simulated aerosol state at the indicated humidity and at 20°C according to the method described by Miller [8]. For this aerosolization the cells were washed from a glass fiber using an 0.8 percent solution of NaCl, centrifuged, and the sediment was resuspended in in 3 ml of the same solution. We measured the concentration of bacterial cells in the suspension with a spectrophotometer at a wave length of 600 nm.

Results and discussion. In previous studies that used the nuclear magnetic resonance method, researchers found evidence of conformational changes in the macromolecules of microbial cells that had been aerosolized in the air; in several cases this caused the death of the bacteria [1]. Methods for determining the hydration water in bacteria were used to reveal these changes. The method developed by Kuntz and Kauzmann, which does not depend on measuring the conformation of the macromolecules, and the method developed by Daszkiewicz et al., in which the amount of hydration water is estimated by using the time of proton relaxation in the cell water, give the same results for cells in suspension. But if significant conformational changes take place in the macromolecules of the aerosolized cells compared to the original conformation, there is a substantial change in the characteristics of proton relaxation in the hydration water. In this case, according to the method developed by Daszkiewicz et al., a value for the amount of hydration water V_{ef} will be obtained that depends on conformational changes in the macromolecules and is distinct from the original value of the amount of hydration water V_k, which was obtained according to the method of Kuntz and Kauzmann. Therefore, the ratio V_{ef}/V_k can serve as a criterion for conformational changes in the macromolecules of aerosolized cells.

Table 1. The change in the V_{ef}/V_k ratio in aerosolized E. coli at different humidity

ОВ. воздуха, %(1)	$V_{эф}/V_к$ при содержании общей воды в клетке (в г воды на 1 г сухой массы) (до)(2)								
	1,5	1,2	0,7	0,6	0,5	0,4	0,35	0,3	0,2
40	1	1	1	1	1	1	1	1	1
55	1	1	1,9	2,2	2,6	3,0	3,4	3,4	3,4
70	1	1	2	2,3	2,6	2,7	2,7	2,7	2,8
90	1	1	0,9	1	1	1	1,1	—	—

Key:

1. Relative humidity of the air, percent

2. V_{ef}/V_k for a cell's given total water content (in grams of water per 1 gram dry mass) (before)

The results of determining the relationship between quantities of hydration water (V_{ef}/V_k) in aerosolized microbial cells are presented in table 1. The ratio V_{ef}/V_k deviates significantly from 1 when the relative humidity is between 55 and 70 percent, after the cells contain less than 1.2 grams of water per 1 g dry mass of bacteria; this is, apparently, evidence of significant denaturational changes and irreversible aggregation. At a relative humidity of 40 or 90 percent, V_{ef}/V_k did not deviate substantially from 1, regardless of the amount of water remaining in the cells; that is, at these levels of humidity there were no significant conformational changes in the macromolecules.

We also confirmed the existence of conformational changes in aerosolized cells using a biochemical method for studying the activity of beta-galactosidase in aerosolized cells at varying relative humidity. This enzyme was chosen as a model for a protein macromolecule because there is a convenient and sensitive method for analyzing its structural integrity [3]. The enzymatic activity of beta-galactosidase was used as the criterion for structural integrity of bacterial macromolecules after aerosolization; this was measured as a percentage of its activity before aerosolization, since it is well known that a change in the activity of an enzyme occurs as a result of conformational changes or destruction of its oligomeric structure. Table 2 contains a summary of the results of measuring the activity of beta-galactosidase in E. coli, aerosolized at relative humidity ranging from 30 to 80 percent. The results show that beta-galactosidase was least active in microbial cells that were aerosolized at a humidity of about 50 percent (this level had the maximum harmful effect); with an increase and decrease in humidity the activity rose and reached its maximum level at a relative humidity of 80 percent, when the survival rate was highest under the experimental conditions. Consequently, the activity of beta-galactosidase corresponds to the survival rate of E. coli at varying levels of humidity.

Table 2. Survival of E. coli and activity of beta-galactosidase after the microorganism is exposed to air of varying humidity for 15 minutes

Показатель (1)	Хранение E. coli (2)			
	в суспензии (3)	в аэрозоле при ОВ (в %) (4)		
		30	50	80
Выживаемость, % (5)	100	10±2	6±1	30±4
Активность галактозидазы, % (6)	100	51,3±2	41±2	61±3

Key:

1. Indicator
2. State of E. coli
3. In suspension
4. In aerosol form at a given relative humidity (in percent)
5. Survival rate (percent)
6. Activity of galactosidase (percent)

Thus, the experiments with beta-galactosidase confirmed that when microbial cells are put into aerosol form there are significant conformational changes in their macromolecules that depend on the rate of dehydration.

The results of our previous study of dehydration in aerosolized cells [1] and data obtained in the present study provide us with a basis for the following description of one of the mechanisms involved in the death of microbial cells under these conditions.

When the relative humidity range is 50-100 percent: As more water is removed from an aerosolized cell, the concentration of proteins and salts in the cell rises. Starting at a certain level of dehydration the proteins undergo conformational changes and aggregation in an aqueous solution. These processes are accompanied by an initial increase in the amount of hydration water in the bacteria. With further dehydration a balance is reached between the atmospheric humidity and the intracellular water. The preservation of or change in the native conformation of macromolecules in bacterial cell and the cell's viability depend on the rate of dehydration and the amount of hydration water that remains.

When E. coli cells are dispersed in air that has a relative humidity of between 70 and 100 percent, the rate of evaporation from the microbial cells is relatively low. After the initial increase, the amount of hydration water in the cells decreases to the level that the cells had in the suspension. All this helps to preserve the native conformation of the macromolecules and the development of reversible processes in those cells in which conformational changes and aggregation took place. Evidence of this can be seen when the value V_{ef}/V_k is close to 1, and also when the indicators of beta-galactosidase activity and the survival of microbial cells in the air are high.

When microbial cells are dispersed into air with humidity between 50 and 70 percent, reversible aggregation and conformational changes occur during the first dehydration period, when the total water content in the cells exceeds 1.2 g of water per 1 g of dry mass. This is indicated by a value of V_{ef}/V_k that is close to 1. Further dehydration of cells, when their total water content drops below 1.2 g of water per 1 g of dry mass, causes irreversible conformational changes in and aggregation of the macromolecules. This is indicated by a value of $V_{ef}/V_k > 1$, a decrease in the activity of beta-galactosidase, and an increase in the rate at which the microbial cells die in the air. Irreversible conformational changes in the macromolecules probably occur after the removal of the free water and part of the hydration water; the volume of hydration water should still be adequate to maintain intramolecular mobility of the macromolecules. In this case, removal of part of the hydration water opens up reaction-capable groups of molecules and preservation of the intramolecular mobility promotes formation of undesirable bonds between these groups, and consequently, irreversible conformational changes and aggregation become possible. Therefore, the largest amount of irreversible conformational changes and aggregation should occur at some critical relationship between the water that has been removed and that which remains, and at some optimal rate of evaporation. Apparently, for E. coli this critical ratio is a relative humidity of 50 percent. We should point out that macromolecules undergo irreversible conformational changes not only at the end

of the dehydration process when all the free water has already been removed, but also earlier on, as a result of irregularity in the dehydration process.

When the relative humidity is between 30 and 40 percent: Apparently, when the relative humidity is 40 percent the rate of dehydration is higher than the critical level and the free water and part of the hydration water are removed so rapidly that the "almost dry" macromolecules aggregate among themselves; the rapid removal of a significant quantity of hydration water causes a sharp decrease in the intramolecular mobility of the macromolecules, which hinders conformational changes and preserves the native conformation of the macromolecules. The small number of conformational changes and the "dry" aggregation are evidenced by the absence of an increase in the amount of hydration water in the microbial cells at the beginning of dehydration, by the value V_{ef}/V_k being close to 1, and a higher level of activity of beta-galactosidase than observed when the relative humidity is 50 percent. Therefore, the smaller number of conformational changes contributes to a survival rate that is higher when the relative humidity is between 30 and 40 percent than when it is 50 percent.

Webb [9] observed a strong survival rate among E. coli at a low humidity in experiments in which a suspension of bacterial cells were dispersed in an atmosphere of nitrogen with differing relative humidity in the absence of air. He showed that the humidity of the air serves as a good protector against the lethal effect of oxygen, which is toxic only when the humidity is low. In our experiments we aerosolized E. coli in a air medium with a relative humidity of 30 percent and with 2 factors operating on the microbial cells--dehydration and the oxygen in the air. Therefore, at a relative humidity of 30 percent we found the survival of the E. coli cells to be lower than it would be as a result of dehydration alone.

The results of the research show that the irreversible conformational changes that occur as a result of rapid dehydration are some of the important lethal processes in aerosolized bacteria. These changes can lead to a disruption in the activity of many enzymes or to a total loss of activity and to unbalanced metabolic processes, which in turn leads to the death of the microbial cells.

One should keep in mind that irreversible conformational changes in bacterial macromolecules can also be caused by rehydration when bacteria are transferred to a fluid medium, but there are still no methods for studying this process, since the bacteria must be resuspended before its viability can be demonstrated.

Conclusions.

1. The activity of beta-galactosidase in aerosolized E. coli cells depends on the moisture in the air and is directly proportional to the viability of the microbial cells under these conditions.

2. The death of E. coli cells in air is caused by irreversible conformational changes and aggregation of their macromolecules.

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QUANTITATIVE CHARACTERISTICS OF SURVIVAL PHENOMENON IN EXPERIMENTAL PLAGUE

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[Article by G. P. Aparin and T. I. Vershinina, Antiplague of Siberia and the Far East Scientific Research Institute, Irkutsk]

[Text] The survival phenomenon described by Ginsburg [3, 4] consists of isolated microbes of the anthrax agent losing their ability to cause this lethal infection in guinea pigs when they are given one injection with 10^5 microbes of the vaccine strain. The survival phenomenon has been studied in experimental plague [5, 6, 8] and tularemia [7]. It is used to prove the harmlessness of live vaccines when there is possible spontaneous reversion of virulence in individual microbes in a population of the vaccine strain.

The aim of the present study was to develop a method for making a quantitative evaluation of the survival phenomenon, and to study its specificity and possible correlation to the immunogenicity of an avirulent strain.

Materials and methods. We used Yersinia pestis strains: the virulent strain was the P1300 (Dcl for white mice with 10^2 microbes and $8 \cdot 10^2$ microbes for guinea pigs); the avirulent strains were the EV NIIEG, the vaccine, P1300 P-, the strain selected from the P1300 strain in a synthetic medium with hemin [11], EV 5 Fl- Pen^r Trp-, a strain obtained from the "Microbe" All-Union Antiplague Institute, 926 Otten, P1300 Ca+, a strain selected from the P1300 strain in a medium with a deficiency of calcium at a culture temperature of 37° [10], I2377 Rha+ Aga-, isolated in the Gorno-Altay plague nidus; strains virulent in white mice but avirulent in guinea pigs were the I2885 Rha+ Aga- from Gorno-Altay and I2785 Rha+ Aga- from northwestern Mongolia. We also used the Yersinia pseudotuberculosis 54, Yersinia entercolitica 104, and Escherichia coli K-12.

A quantitative evaluation of the effect of the survival phenomenon was made as follows. Mixed-breed white mice were infected with a mixture of suspensions of virulent and avirulent strains in a physiological solution, using a subcutaneous injection of 0.5 ml. The suspensions were diluted so that each mixture contained 10^7 microbes of the avirulent strain and the number of microbes of the virulent strain was equal to the Dcl (1, 2, 3, 4, Dcl, etc.) The white mice infected with each mixture were divided into groups depending on the Dcl number of each virulent strain. Each group contained 10 animals. Between 60 and 100 mice were used to test each avirulent strain. In each

experiment 10 white mice were used for the control value of the Dcl of the P1300 strain, and the avirulence of the corresponding strain was tested by infecting 10 white mice with a dose of 10^7 microbes. The infected animals were observed for 21 days. The test results were used to calculate the LD_{50} indicator of the virulent strain introduced with the avirulent strain, and it was described by means of the Dcl number. The greater the survival, the higher the LD_{50} number. The ED_{50} was determined to describe the immunogenicity of the avirulent strains of the plague microbe [2], using 40 white mice for each strain.

In the experiments with guinea pigs the LD_{50} was determined using the same methods. The dose of avirulent culture in the mixture was 10^9 microbes. The mixture of cultures was introduced subcutaneously in each animal in a 1 ml physiological solution. Forty guinea pigs were infected with a suspension of each strain with a chosen virulence mixed with a culture of the P1300 strain.

In all the experiments the LD_{50} and ED_{50} indicators were analyzed statistically [9].

Results and discussion. Table 1 contains the LD_{50} indicators of the P1300 strain introduced into white mice in a mixture with strains of avirulent plague microbes, Yersinia, and intestinal bacilli; and the ED_{50} indicators that describe the immunogenicity of avirulent strains of plague agents.

When using different avirulent strains to reproduce the survival phenomenon, different numbers of white mice died, which was reflected in the LD_{50} indicators of Y. pestis P1300. The highest values for LD_{50} were obtained when the strains of P1300 P- and EV NIIEG were used. The LD_{50} indicators obtained in the tests with these strains differed substantially from the corresponding indicators in other tests. Low ED_{50} values correspond to high LD_{50} values. The ED_{50} values do not exceed 10^4 microbes, which is evidence of the immunogenicity of the P1300 P- and EV NIIEG strains [2]. The ED_{50} indicator in the I2377 strain with a lower immunogenicity is 4.7 times higher than in the Y. pestis EV NIIEG, while the LD_{50} indicator in reproducing the survival phenomenon with the given strain is ten-sixtythirds of the corresponding indicator in the experiment with the EV NIIEG strain. In the latter case the difference is statistically significant.

In reproducing the survival phenomenon in experiments with nonimmunogenic strains of the plague microbe (EV 5, 926, P1300 Ca+), a significant number of white mice died; low values for LD_{50} contributed to this. The ED_{50} indicators for these strains are very high.

Therefore, when reproducing the survival phenomenon the LD_{50} indicator of the virulent strain can serve as an accurate criterion for the immunogenicity of the avirulent strain. The advantage of using the LD_{50} criterion is that it cuts in half the time needed to determine the immunogenicity and the two-stage infection of the animals can be replaced by a one-stage infection process.

The virulent strain of P1300 is genetically similar to the avirulent strains P1300 P- and P1300 Ca+. The LD_{50} indicators in tests with the two latter strains differed significantly ($P<0.001$). In the process of population

variability in a culture of the P1300 strain, cells appear with properties of the P1300 P- and P1300 Ca+ strains. When the avirulent immogenic P- cells accumulate they can reduce the virulence of the original strain significantly, while the avirulent immunogenic Ca+ cells have a very weak effect on the virulence of the original strain.

Table 1. Quantitative evaluation of the survival phenomenon in white mice

Штамм (1)	Показатель LD_{50} (в Dcl для белых мышей) Y. pestis P1300, введенного в смеси с другими штаммами, и пределы его колебаний (2)	P по отношению к результату опыта с использованием Y pestis EB НИИЭГ (3)	Иммуногенность штаммов Y. pestis (4)	
			показатель ED_{50} и пределы его колебаний (число микробов) (5)	P по отношению к ED_{50} Y. pestis EB НИИЭГ (6)
Y. pestis EB НИИЭГ (7)	233,8 (171,4—318,4)	—	7 478 4 157—13 450	—
Y. pestis P1300 P—	>256,0	>0,05*	1 994 128—3 185	>0,05
Y. pestis И2377 (8)	36,8 (26,7—50,7)	<0,01	35 230 17 320—76 630	>0,05
Y. pestis EB 5 (EV 5)	6,1 (4,5—8,4)	<0,001	350 100 184 000—666 500	<0,01
Y. pestis 926 Оттена (9)	3,3 (2,5—4,4)	<0,001	>3 125 000	<0,001**
Y. pestis P1300 Ca+	2,6 (2,2—3,3)	<0,001	>3 125 000	<0,001**
Y. pseudotuberculosis 54	5,1 (3,7—7,2)	<0,001	Не изучалась (10)	
Y. enterocolitica 104	2,5 (2—3,3)	<0,001	» »	
E. coli K-12	1,5 (1,3—1,8)	<0,001	» »	

* Compared to LD_{25} in corresponding experiments
** Compared to ED_{25} from the ED_{25} in the EV NIIEG strain

Key:

1. Strain
2. The LD_{50} indicator (in Dcl for white mice) for Y. pestis P1300, introduced in a mixture with other strains, and the limits of its fluctuations
3. P in terms of the result of the experiment using Y. pestis EV NIIEG
4. Immunogenicity of Y. pestis strains
5. The ED_{50} indicator and the limits of its fluctuations (number of microbes)
6. P in terms of ED_{50} in Y. pestis EV NIIEG
7. Y. pestis EV NIIEG
8. Y. pestis I2377
9. Y. pestis Otten 926
10. Not studied

The survival phenomenon was also obtained when yersinoses and intestinal bacillus agents were used as the avirulent cultures, and the LD_{50} indicators were low. They differed significantly from the corresponding indicators in the EV NIIEG and P1300 P- immunogenic strains of the plague microbe. However, when comparing the results of the tests using Y. pseudotuberculosis 54, and Y. enterocolitica 104 with the results of tests in which nonimmunogenic strains of plague agents were used, there were no significant differences in the LD_{50}

values (P>0.05). The LD_{50} indicator obtained in the experiment with intestinal bacillus differed significantly from the analogous indicator obtained when Y. pestis EV 5, Y. pseudotuberculosis 54, and Y. pestis 926 were used, but it did not differ significantly from the LD_{50} indicator obtained in experiments using Y. pestis P1300 Ca+ and Y. enterocolitica 104.

Therefore, the survival phenomenon is not strictly specific. It is also reproduced with heterogenous avirulent cultures. Under natural conditions it is likely that animals are infected by nonpathogenic microflora. Apparently in these cases the effect of the survival phenomenon will hinder the development of the plague infection.

There is better reproduction of the survival phenomenon with plague in white mice than in guinea pigs [1]. Tests were conducted on guinea pigs to study the ability of the strains with selective virulence to elicit the survival phenomenon in animals not sensitive to those strains. The test results are presented in table 2.

Table 2. A quantitative evaluation of the survival phenomenon in guinea pigs

Штамм (1)	(2) Показатель LD_{50} (в Dcl для морских свинок) Y. pestis P1300, введенного в смеси с другими штаммами и пределы его колебания	P по отношению к результату опыта с использованием Y. pestis EB НИИЭГ (3)
Y. pestis EB НИИЭГ (4)	0.59 (0.46–0.76)	—
Y. pestis I2885 (5)	0.85 0.64–1.12	>0.05
Y. pestis I2785 (6)	0.34 0.24–0.44	>0.05

Key:

1. Strain
2. LD_{50} indicator (in Dcl for guinea pigs) Y. pestis P1300, introduced in a mixture with other strans and the limits of its fluctuation
3. P in terms of the result of the experiment using Y. pestis EV NIIEG
4. Y. pestis EV NIIEG
5. Y. pestis I2885
6. Y. pestis I2785

By comparing the data in tables 1 and 2, we see that the quantitative indicators of the survival phenomenon in experiments with the EV NIIEG strains in white mice are 396 times higher than in guinea pigs.

In the experiments with the I2885 and I2785 strains the quantitative indicators of the survival phenomenon were practically the same as in the experiment with the EV NIIEG strain (P>0.05). The strains that were avirulent in guinea pigs

in a number of cases turned out to be capable of preventing the development of a plague infection cuased by strains with a universal virulence.

Conclusions

1. We have suggested a method for making a quantitative evaluation of the effect of the survival phenomenon by determining the LD_{50} indicator of the virulent strain introduced in white mice or guinea pigs in a mixture with avirulent strains.

2. In experiments on white mice, a clear inverse correlation was traced between the ED_{50} indicators of avirulent strains of the plague microbe used to reproduce the survival phenomenon and the criterion for LD_{50} that describes the effect of this phenomenon. The latter can be used for an accelerated definition of the immunogenicity of avirulent strains of the plague agent.

3. A low level of specificity was demonstrated for the survival phenomenon in the presence of plague. Cultures of yersiniosis agents did not differ from avirulent nonimmunogenic strains of the plague microbe in terms of the quantitative characteristics of the survival phenomenon that they cause.

4. The accumulation of immunogenic avirulent cells in a culture of a virulent strain of a plague agent decreases its pathogenic action more than the accumulation of nonimmunogenic avirulent cells.

5. We have confirmed data on the relatively weak effect of the survival phenomenon in guinea pigs. Strains with a selective virulence for white mice elicit a survival phenomenon effect in guinea pigs with the same quantitative characteristics as the EV NIIEG strain.

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ATTEMPT AT REVEALING HOST SPECIFICITY SYSTEMS IN YERSINIA PESTIS

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[Article by G. V. Demidova, V. P. Zyuzina, and V. I. Tynyanova, Antiplague Scientific Research Institute, Rostov on Don]

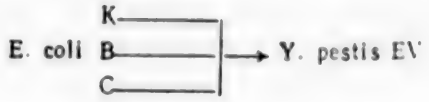
[Text] We made an attempt earlier to reveal the host specificity systems in various strains of plague agents. We tested modification-restriction (m-r) enzymes using a mild H bacteriophage [2]. When working with this method we did not see the action of the restriction endonucleases either in cross titration of the phage between the Yersinia pestis strains EV, 1, 17, 556, 1300, 773 or when infecting them with the phage modified by the m-r systems of Escherichia coli type K, B, RI and RII. Since the restriction activity is, as a rule, used to determine the presence of the m-r enzymes in bacteria, (methylases do not have their own selection methods), it would be logical to suppose that the strains of Y. pestis being studied are without host specificity. However, for a final conclusion it was necessary to confirm the results that were obtained using other experimental approaches. With this aim, in the present study we used conjugation and transformation methods. The success of transfer of plasmid DNA using these methods depends on several factors: the compatibility of specific genomes in the cell, the recipient bacteria overcoming the barrier of the cell wall, the action of the restriction endonucleases, and the possibility for expression of genes that are being carried by the given plasmid. When there is proper matching of partners in the crossing, the number of limiting factors is reduced to one--the action of the restriction endonucleases. In this case the frequency of a plasmid's transfer can serve as a measure of identity of the m-r systems of its previous host and its present host.

Materials and methods. In this study we used strains of the E. coli bacterial bacilli (which were kindly provided by Ya. I. Bur'yanov of the Biochemistry and Physiology of Microorganisms Institute of the USSR Academy of Sciences, Pushchino); E. coli C600 thr, leu, thi, lac, $r+_k m+_k$; E. coli 803 gal, met, $r-_k m-_k$; E. coli 707 gal, met, $r+_B m+_B$; E. coli 834 gal, met, $r-_B m-_B$, Y. pestis EV 1290, conjugated plasmid R222Cm, Sm, Su, Tc; and unconjugated plasmid RSF 2121 Ap. The R222 plasmid was conjugated with Y. pestis and E. coli. When the recipient was the Y. pestis EV 1290 strain, and the donors were E. coli strains with different m-r systems, fluid cultures of donor and recipient bacteria were mixed at a ratio of 1:5. Selection was performed on the basis of resistance to

tetracycline and counterselection was done on the basis of resistance to polymyxin. In the experiments in which the Y. pestis EV 1290 strain was the donor, the donor-recipient ratio was 2:1. Selection was based on resistance to tetracycline and counterselection was based on resistance to rifampicin. In both cases the crossing was done at 37°C over the course of 3 hours. The effectiveness of conjugation was determined as the ratio between the number of transconjugates and the number of viable cells of the recipient in 1 ml of the reaction mixture.

The DNA of the RSF plasmid from E. coli and Y. pestis was determined according to the method described by Guerry [6] and further purification was done in a density gradient of cesium chloride with ethyl bromide. Transformation was performed on recipient cells of E. coli and Y. pestis EV, treated in 2 different ways: by calcium chloride [5] and freezing and thawing [3]. The effectiveness of the DNA transfer per cell was evaluated as the ratio between the number of transformants and the total number of viable bacteria per 1 ml of the mixture.

Results and discussion. In order to determine whether bacteria of the plague microbe are capable of distinguishing the type of modification in foreign DNA, the conjugative plasmid R222 was transferred to Y. pestis EV cells according to the following system:



This particular arrangement made it possible to compare the frequency of the transfer of the R222 plasmid from each strain of E. coli with the known system to the m-r strain of Y. pestis EV. Table 1 contains the results of these experiments and shows that the effectiveness of the transfer of the R222 plasmid in the E. coli-Y. pestis system in the different series of experiments ranges from $8 \cdot 10^{-7}$ to $1 \cdot 10^{-5}$. An analysis of the data from each specific experiment shows that there is no definite correlation between the frequency of the formation of transconjugates and the type of system of the m-r donor; that is, the Y. pestis EV bacteria are not able to distinguish the type of modification of foreign DNA.

The plasmid was transferred from Y. pestis EV to E. coli only in strains that were without m-r: E. coli C, E. coli 834 and 803 ($r^-_k m^-_k$), as shown in table 2. If E. coli C600 ($r^+_k m^+_k$) and E. coli 707 ($r^+_B m^+_B$) served as recipients, no transconjugates were obtained.

In testing m-r enzymes using the conjugation method to prove the presence of these enzymes in a cell, the thermal treatment method is used very frequently on recipient bacteria. Heating cells at 50°C for 2-3 minutes leads to a suppression of the m-r enzymes' activity, as a result of which the effectivenss of transfer of the plasmid genome into the cells increases significantly in comparison with the control variant [7].

Table 1. Frequency of conjugation transfer of the R222 plasmid from the E. coli C, C600 and 707 strains to the Y. pestis EV strain.

№ опыта (1)	E. coli C × Y. pestis EV		E coli C600 ($r_K^+ m_K^+$) × Y. pestis EV		E. coli 707 ($r_K^+ m_B^+$) · Y. pestis EV	
	число трансконъюгантов (2)	эффективность конъюгации (3)	число трансконъюгантов (4)	эффективность конъюгации (5)	число трансконъюгантов (6)	эффективность конъюгации (7)
1	8	$8 \cdot 10^{-7}$	6	$6 \cdot 10^{-7}$	26	$2{,}6 \cdot 10^{-6}$
2	8	$8 \cdot 10^{-7}$	51	$5 \cdot 10^{-6}$	27	$2{,}7 \cdot 10^{-6}$
3	10	$1 \cdot 10^{-6}$	51	$5 \cdot 10^{-6}$	37	$3{,}7 \cdot 10^{-6}$
4	15	$1{,}5 \cdot 10^{-6}$	51	$5 \cdot 10^{-6}$	38	$3{,}8 \cdot 10^{-6}$
5	15	$1{,}5 \cdot 10^{-6}$	77	$7{,}7 \cdot 10^{-6}$	38	$3{,}8 \cdot 10^{-6}$
6	18	$1{,}8 \cdot 10^{-6}$	77	$7{,}7 \cdot 10^{-6}$	39	$3{,}9 \cdot 10^{-6}$
7	21	$2 \cdot 10^{-6}$	77	$7{,}7 \cdot 10^{-6}$	41	$4 \cdot 10^{-6}$
8	23	$2{,}3 \cdot 10^{-6}$	77	$7{,}7 \cdot 10^{-6}$	48	$4{,}8 \cdot 10^{-6}$
9	28	$2{,}8 \cdot 10^{-6}$	84	$8{,}4 \cdot 10^{-6}$	49	$4{,}9 \cdot 10^{-6}$
10	78	$7{,}8 \cdot 10^{-6}$	89	$8{,}9 \cdot 10^{-6}$	49	$4{,}9 \cdot 10^{-6}$
11	78	$7{,}8 \cdot 10^{-6}$	101	$1 \cdot 10^{-5}$	51	$5{,}1 \cdot 10^{-6}$
12	93	$9{,}3 \cdot 10^{-6}$	102	$1 \cdot 10^{-5}$	51	$5{,}1 \cdot 10^{-6}$
13	107	$1 \cdot 10^{-5}$	110	$1{,}1 \cdot 10^{-5}$	54	$5{,}4 \cdot 10^{-6}$
14	111	$1{,}1 \cdot 10^{-5}$	115	$1{,}2 \cdot 10^{-5}$	54	$5{,}4 \cdot 10^{-6}$
15	121	$1{,}2 \cdot 10^{-5}$	125	$1{,}25 \cdot 10^{-5}$	67	$6{,}7 \cdot 10^{-6}$

Note: The effectiveness of the conjugation is based on $1 \cdot 10^7$ viable cells. The statistical analysis of the results was done according to the method for evaluating the sign criterion [1].

Key:

1. Experiment number
2. Number of transconjugates
3. Effectiveness of conjugation
4. Number of transconjugates
5. Effectiveness of conjugation
6. Number of transconjugates
7. Effectiveness of conjugation

Table 2. Frequency of conjugation transfer of plasmid R222 from the Y. pestis EV strain to the E. coli strains C600 ($r+_k m+_k$), 707 ($r+_k m+_B$), C, 803 ($r-_B m-_k$), ($r-_B m-_B$)

№ опыта (1)	(2) Эффективность передачи плазмиды R222 от штамма Y. pestis EV штаммам:				
	E. coli C	E. coli 834	E. coli 803	E. coli C600	E. coli 707
1	$2 \cdot 10^{-9}$	$4 \cdot 10^{-9}$	$1{,}2 \cdot 10^{-7}$	0	0
2	$4 \cdot 10^{-9}$	$3 \cdot 10^{-9}$	$5 \cdot 10^{-7}$	0	0
3	$2 \cdot 10^{-9}$	$5 \cdot 10^{-9}$	$3 \cdot 10^{-7}$	0	0

Key:

1. Experiment number
2. Effectiveness of transfer of the R222 plasmid from the E. coli strain to the Y. pestis EV strain.

In our experiments the heat treatment applied to the bacteria did not increase the frequency of the transfer of the R222 plasmid into cells of the Y. pestis EV 1290 strain (table 3).

Table 3. The effectiveness of the transfer of the R222 plasmid from the E. coli C600 ($r^+_k m'_k$) [as published] strains into the Y. pestis EV strains before and after heat treatment

(1) Исследование	Температура, °С (2)	Время обработки, мин (3)	Эффективность конъюгации (4)
Контроль (5)	20	10	$1 \cdot 10^{-6}$
Опыт № 1 (6)	50	2	$2{,}5 \cdot 10^{-6}$
	50	10	$2 \cdot 10^{-6}$
Контроль (7)	20	10	$5{,}5 \cdot 10^{-6}$
	50	2	$1{,}5 \cdot 10^{-5}$
Опыт № 2 (8)	50	10	$5 \cdot 10^{-6}$
Контроль (9)	20	10	$1 \cdot 10^{-6}$
	50	2	$8{,}5 \cdot 10^{-6}$
Опыт № 3 (10)	50	10	$2 \cdot 10^{-6}$

Key:

1. Group being studied
2. Temperature, °C
3. Duration of heat treatment, min
4. Effectiveness of conjugation
5. Control
6. Experiment No 1
7. Control
8. Experiment No 2
9. Control
10. Experiment No 3

We also made an attempt to reveal the host specificity system in Y. pestis using the nonconjugative plasmid RSF 2124 and the transformation method. In one series of experiments we used the following pattern:

E. coli C600 (RSF 2124)
Y. pestis EV (RSF 2124) → Y. pestis EV,

and in the other series we used this pattern:

E. coli C600 (RSF 2124)
Y. pestis EV (RSF 2124) → E. coli C600.

Tables 4 and 5 contain the results that were obtained. As table 4 shows, the RSF 2124 plasmid that was isolated from Y. pestis EV and E. coli C600 transfers to the Y. pestis EV bacteria with the same frequency. At the same time, the effectiveness of the transformation of the E. coli C600 DNA of the RSF 2124 plasmid, isolated from the E. coli C600, is significantly higher than the transfer frequency of the same DNA isolated from Y. pestis (see table 5).

Table 4. A comparison of the effectiveness of the transfer of the DNA of plasmid RSF 2124, isolated from the Y. pestis EV and E. coli C600 bacteria

(1) № опыта	Число трансформантов (2)		
	ДНК плазмиды RSF 2124 из Y. pestis EV (3)	ДНК плазмиды RSF 2124 из E. coli C600 (4)	$\frac{\sqrt{n_1}-\sqrt{n_2}}{\text{или}} > 1,4$
1	113	118	0,3 <1,4
2	58	73	0,9 <1,4
3	43	33	0,9 <1,4
4	12	14	0,2 <1,4

Note: Here and in table 5: The number of transformants is calculated per $1 \cdot 10^8$ viable cells of Y. pestis EV; the results were analyzed statistically.

Key:

1. Experiment number
2. Number of transformants
3. The DNA of the RSF 2124 plasmid from Y. pestis EV
4. The DNA of the RSF 2124 plasmid from E. coli C600

Table 5. A comparison of the effectiveness of the transfer of DNA of the RSF 2124 plasmid, isolated from the Y. pestis EV and E. coli C600 bacteria, to the E. coli C600 strain

(1) № опыта	Число трансформантов (2)		
	ДНК плазмиды RSF 2124 из Y. pestis EV (3)	ДНК плазмиды RSF 2124 из E. coli C600 (4)	$\frac{\sqrt{n_1}-\sqrt{n_2}}{\text{или}} > 1,4$
1	23	284	12 >1,4
2	52	600	17,3 >1,4
3	113	1981	33,9 >1,4
4	202	2005	30,6 >1,4

Key:

1. Experiment number
2. Number of transformants
3. The DNA of the RSF 2124 plasmid from Y. pestis EV
4. The DNA of the RSF 2124 plasmid from E. coli C600

Thus, using the methods of conjugation and transformation, we encountered the same situation as in the earlier research using the H bacteriophage: we were not able to detect any host specificity systems in the Y. pestis EV No 1, 17, 556, 1300, and 773 strains.

Conclusion

No host specificity systems were found in the Y. pestis EV, No 1, 17, 556, 1300, and 773 strains.

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ISOLATION OF TYPE B STAPHYLOCOCCAL ENTEROTOXIN AND STUDY OF SOME OF ITS PHYSICO-CHEMICAL, BIOLOGICAL AND IMMUNOLOGICAL CHARACTERISTICS

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 (manuscript received 16 Nov 82) pp 36-41

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[Text] Six types of staphylococcal enterotoxins are known (A, B, C, D, E, F); they differ in terms of antigenic specificity and their physico-chemical and biological characteristics. The interest in studying enterotoxins and in obtaining them in a homogeneous form has been generated by a desire to use them to produce antitoxic sera, which are used extensively in developing specific, highly sensitive immunological methods for typing staphylococci and for diagnosis of food poisoning. In addition to this, recently staphylococcal enterotoxins have been used as inducers of immune interferon.

Several methods consisting of 4-5 stages have been proposed for isolating staphylococcal enterotoxins [18], however, even when these methods are used it is not always possible to obtain a homogeneous preparation of enterotoxins [13]. We proposed a simpler 3-stage method for isolating type A staphylococcal enterotoxin [4]. There is no doubt about the need to develop simpler methods for isolating other types of staphylococcal enterotoxins.

It should be pointed out that staphylococcal enterotoxin proteins, in spite of their common biological action, differ in terms of several characteristics. A significant variation in physico-chemical, biological, and immunological properties has been noted depending on the producer-strain and the isolation and purification methods used. Several values have been found for the molecular weight of the type B enterotoxin, ranging from 24,000 to 35,000 [12, 20]. No single opinion has been reached regarding the isoelectric point for the type B enterotoxin. Metzger et al. [15] found that the pI of the type B enterotoxin that they isolated was 8.6; Chang and Dickie's data [9] show that there are several forms of this toxin with pI values of 7.8, 8.0, 8.25, and 8.55. There are also extremely contradictory data regarding the stability of staphylococcal enterotoxins in the presence of urea [3, 7, 21] and the minimum toxic dose of enterotoxins isolated by means of various methods.

We set ourselves the task of obtaining a homogeneous preparation of type B staphylococcal enterotoxin (SEB); describing some of its physico-chemical,

biological, and immunological properties; and obtaining a monospecific enterotoxin type B serum that is necessary for identifying an enterotoxin using immunological methods.

Materials and methods. We used the following strains: S6 715H Staphylococcus aureus with an elevated production of the type B enterotoxin (from Dr. Kondo, Japan); S. aureus FRI 722 (England) which produces the type A toxin; 493 which produces the type C toxin; 494 which produces the type D toxin; 326 which produces the type E toxin (from Professor M. S. Bergdolla, USA); and the Wood-46 (from the Standardization and Control of Medical Biological Preparations State Scientific Research Institute imeni L. A. Tarasevich). Earlier we obtained type A and C staphylococcal enterotoxins and antisera for them [4-6]. We used the type B anti-enterotoxin staphylococcal serum from the "Serva" company as a reference serum.

In order to obtain the enterotoxin we grew bacteria in matrices with 4 l of Casman's sterile nutrient medium [8] with our own modification. The modification consisted of replacing the casein hydrosylate from the "Difco" company with an enzymatic casein hydrosylate produced by the Epidemiology and Microbiology Institue imeni N. F. Gamaleya of the USSR Academy of Medical Sciences, and adding 1 percent yeast extract. After seeding the daily culture removed from the tapered agar to the matrices, the bacteria were incubated at 37°C for 10 hours with aeration. The culture fluid, which was separated from the microbial cells by centrifugation, was diluted with water that had been distilled 3 times and a pH of 6.4 was established. Culture fluids that provided positive reactions with a homologous antiserum and that had biological activity when used in studies on cats, were used in this work.

In order to adsorb the enterotoxin from the culture fluid we used Amberlit CG-50 type II (100-200 mesh) from the "Mallinckrodt Chemical Works." We treated the resin with 0.5 N NaOH for 30 minutes and then rinsed it with distilled water to bring it to a pH of 8.0; it was then treated with 0.5 N HCl for 30 minutes and rinsed with distilled water to bring it to a pH of 6.0; it was equilibrated in a 0.05 M solution of NaH_2PO_4 with a pH of 6.4.

We determined the protein according to the method described by Lowry et al. [14], and also by using a spectrophotometer; we determined the nitrogen according to the method described by Grishchenko [2].

We performed disc electrophoresis in polyacrylamide gel at a pH of 4.3 using the method described by Reisfeld et al. [17] and at a pH of 8.6, we used Davis' method [10]. The quantity of protein taken for analysis was 15-25 micrograms per tube. We used blue coumassy for the stain and the gels were rinsed with an acetic acid solution.

We used the method described by Weber and Osborn [22] to determine the molecular weight of the type B enterotoxin. For markers we used transferrin with a molecular weight of 88,000; bovine albumin (with a molecular weight of 67,000); egg albumin (with a molecular weight of 45,000); and myoglobin (with a molecular weight of 17,800).

The material for the electrofocusing was concentrated using an "Amicon" type concentrator with a UM-10 membrane.

The isoelectric focusing was done in a 8101 type tube with a 110 ml capacity (LKB, "Producter AB","Bromma", Sweden) in a gradient of 2 percent ampholins with a pH range of 7.0 to 9.0 and 0-40 percent sucrose according to the method described by Vestberg and Svensson [19] for 96 hours at 600 volts and for 5 hours at 1000 volts. The ampholins were removed by gel filtration in a tube with Sephadex G-25. The hydrogen ion concentration was determined on a TTT-1 potentiometer (Denmark). The biological activity of the preparations was tested on cats weighing between 3 and 3.5 kg according to the method described by Clark and Page. Enterotoxic activity was described as the minimum enterotoxic dose, which is equal to the minimum quantity of material that will cause emesis in the animals. An immunochemical study of the enterotoxin was made according to the method of double diffusion in gel [16] and the immunoelectrophoresis method [1]. The hemolytic activity of the preparations was determined according to the method described by Kondo et al. [13].

Results and discussion. During the first stage of the study we solved the problem of concentrating the enterotoxin. To do this, many investigators have used polyethylene glycol, as well as lyophilization. However, when working with large quantities of the culture filtrate, a number of problems are encountered when these methods are used. In order to concentrate and isolate the native culture material, we used the first stage of the sorption method on Amberlite CG-50 described by Schantz et al. [18], with our own modification. The modification of the first stage of Schantz' method consisted of adding a double quantity of the resin (4 g per 1 l) to the diluted culture material; the mixture was stirred vigorously at room temperature for 1 hour; the resin was separated and rinsed with distilled water from the unabsorbed material in a Büchner funnel. The thoroughness of the rinsing was controlled by readings from the spectrophotometer (E_{280nm}). The resin and the enterotoxin absorbed on it were placed in a 2.5 x 40 cm tube. The elution was performed using a 0.5 M solution of NaH_2PO_4 containing 0.25 M NaCl with a pH of 6.8. The fluid was flowing out at a rate of 2-3 m//min; and 10 ml of fractions were collected on the chromatographic collector. The distribution curve constructed for the adsorption of the solution at E_{280nm} offers evidence of the division of the material into 2 fractions (figure 1). The solutions of the first and second peaks, with adsorption over 1.5, were combined into the first and second fractions. Both fractions were dialyzed for 24 hours with distilled water, after which we determined their protein content, total nitrogen content, and their biological activity in cats. We established that only the fraction corresponding to the first peak had any enterotoxic activity. The minimum enterotoxic dose of this fraction for cats was 50 micrograms of protein per 1 kg body weight (table 1). This is one-fortieth the dose of the original material. This fraction had a positive precipitation reaction with the serum for SEB produced by the "Serva" company (figure 2) [not reproduced]. The second fraction with CG-50 did not demonstrate any enterotoxic activity when introduced into cats in a dose of 100 micrograms per 1 kg body weight.

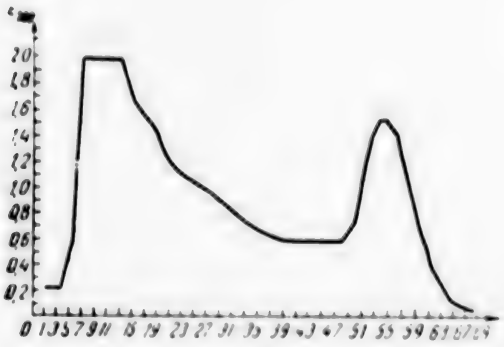

Figure 1. Fractionation of type B staphylococcal enterotoxin in a tube with CG-50. The horizontal axis represents the number of the test tubes.

Table 1. The biological activity of type B staphylococcal enterotoxin

Препарат (1)	Общее количество белка, мг (2)	МЭД в 1 мг белка (3)	Общее число МЭД (4)	Выход МЭД, % (5)
Исходный культуральный фильтрат (6)	12 000	2,0	6 000	100
1-я фракция с CG-50 (после диализа) (7)	200	0,050	4 000	66
2-я фракция с электрофокусировки (pI 8,6) (8)	6	0,003 0,005	2 000	33,0

Key:

1. Preparation
2. Total amount of protein, mg
3. Minimum enterotoxic dose in 1 mg of protein
4. Total number of minimum enterotoxic doses
5. Yield of the minimum enterotoxic dose, percent
6. Original culture filtrate
7. First fraction with CG-50 (after dialysis)
8. Second fraction from electrofocusing (pI 8.6)

We should emphasize that in the dialysis of the first and second fractions with CG-50 there was removal of low molecular inert nitrous components, which made up 70-80 percent of the first fraction and 87-90 percent of the second component.

Because of the yield of the enterotoxin in the first fraction, it was used for further research. Disc electrophoresis in polyacrylamide gel showed that the first fraction of SEB with CG-50 contains 4 components, in contrast to the original culture filtrate with 12-14 components (figure 3) [not reproduced].

In the next stage this fraction with CG-50 underwent electrofocusing. At the end of the experiment the contents of the tube were fractionated on a collector with control over the optic density (E_{280}) and the pH. As figure 4 shows, the material subjected to electrofocusing separated into 2 fractions according to the data on optic density and the curve of the pH gradient. One fraction was focused at a pH of 7.5 and the other at a pH of 8.6. In order to remove the ampholines both fractions were passed through a column of Sephadex G-25 (1.5 x x 100 cm).

The results of the tests on the biological activity of the fractions showed that only the fraction with a pI of 8.6 had any enterotoxic activity (see table 2). The minimum enterotoxin dose of this fraction was 3-5 micrograms of protein per 1 kg body weight, which is one-four-hundredth of the dose of original material (see table 2). The yield of active material was 33 percent.

Table 2. The biological activity of preparations of type B staphylococcal enterotoxin at various stages of purification

Препарат (1)	(2) Количество вводимого белка, мкг на 1 кг массы кошки	(3) Число животных в опыте (числитель) и число реагировавших животных (знаменатель)
Культуральный фильтрат (4)	2000	3/3
	1000	2/0
1-я фракция СЭВ с CG-50 (5)	50	3/3
	25	3/0
2-я фракция с CG-50 (6)	2000	3/2
	300	2/1
	100	3/0
1-я фракция с электрофокусирования (pI 7,25) (7)	2000	2/1
	1000	3/0
2-я фракция с электрофокусирования (pI 8,6) (8)	300	4/4
	150	3/3
	50	3/3
	25	3/3
	10	4/4
	5	5/5
	3	3/2
	1	3/0

Key:

1. Preparation
2. Amount of protein introduced, expressed as µg per kg of cat's body weight
3. Number of animals in experiment (numerator) and number of animals that reacted (denominator)
4. Culture filtrate
5. First SEB fraction from CG-50
6. Second fraction from CG-50
7. First fraction from electrofocusing (pI 7.25)
8. Second fraction from electrofocusing (pI 8.6)

The SEB preparation was not found to contain any l- or ß-hemolysins. The SEB fraction at pI 8.6 in disc electrophoresis contained one component (see figure 3, D). Our obtaining as a result of electrofocusing just one fraction with pI 8.6 and with enterotoxic activity corresponds to the data obtained by Lowry et al. [14]. However, in experiments in which more purified material was used in electrofocusing, the SEB focused in 3 fractions with pI 8.0, 8.25, and 8.6 (table 3). The fractions were immunochemically identical and had a molecular weight of 28,000.

Table 3. Some properties of purified type B enterotoxin

External appearance	White fluffy powder
Solubility	Dissolves well in water and saline solutions
Thermostability	Thermostable
Molecular weight	28,000 daltons
Number of components in electrophoresis in polyacrylamide gel	1
Antigenicity	Pronounced
Number of components in a double diffusion reaction in gel	1
Maximum adsorption	270 nm
Isoelectric point	8.6
Heterogenicity of SEB in terms of pI	The main component--8.6; the minor components--8.0 and 8.25
Antigenic similarity	Has no common antigens with enterotoxins A, C, D, and E
The effect of 6 M urea	The biological activity disappears but the antigenic activity remains
Toxicity in cats (maximum enterotoxin dose)	5 μg per kg of cat's body weight

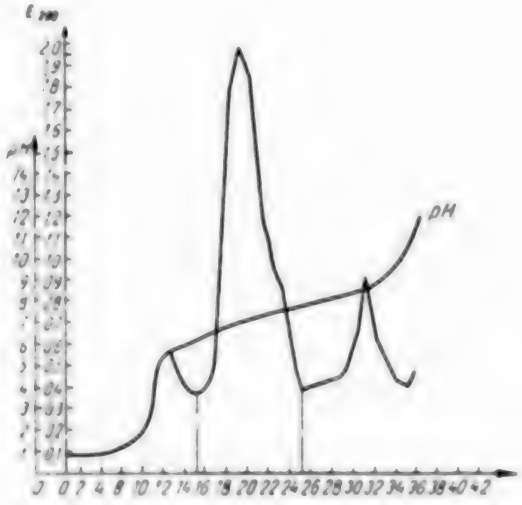

Figure 4. Isoelectric focusing of SEB with 2 percent ampholines in a pH range of 7.0-9.0. The horizontal axis represents the test tube number

When comparing the activity of the isolated SEB with the activity of the preparations obtained by other investigators, one can see that the size of the minimum enterotoxin doses in each instance varies depending on the type of animal and apparently also on the method used to obtain the preparation. The minimum enterotoxin dose for monkeys ranges from 0.1 to 1.6 μg per kg of the animal's body weight [11]. Cats are apparently less sensitive to the enterotoxin; therefore, the minimum enterotoxin dose for cats is somewhat higher--between 0.1 and 5 μg of protein. The results obtained in testing SEB on cats are characterized by a similar value. We showed that the biological

activity of SEB was destroyed by the effect of 6 M urea over the course of 24 hours at 25°C; however, the antigenic activity remained. This demonstrates that different parts of the molecule are responsible for its antigenic and toxic properties (see table 3). We also showed that the toxin's biological activity remained after it was heated for 30 minutes at 60°C, which demonstrates the thermostability of SEB. Using the method of disc electrophoresis in polyacrylamide gel in the presence of SDS, we determined the molecular weight of SEB with pI 8.6. It was 28,000 (figure 5). This value corresponds to the data obtained by other authors [12]. SEB has an ultraviolet absorption spectrum that is characteristic of proteins (see table 3).

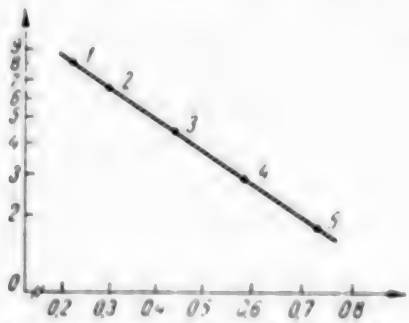

Figure 5. Determination of the molecular weight of SEB using the method of disc electrophoresis in polyacrylamide gel. 1--transferrin (88,000); 2--bovine albumin (67,000); 3--egg albumin (45,000); 4--type B staphylococcal enterotoxin; 5--myoglobin (17,800). The horizontal axis represents the relative mobility of the proteins; the vertical axis represents lg of the proteins' molecular weight.

We studied the antigenic properties of SEB. We obtained an antiserum against SEB in rabbits. The preparation was mixed 1:1 with Freund's adjuvant and was introduced in the animal's paw pads. The amount of SEB in each successive dose increased according to the following model: 0.1, 0.4, 1, 2, 4, 10, 20, 50, 100, 250, and 540 μg of protein . The interval between injections was 6-7 days. One week following the end of the immunization cycle antibodies were found in the animals' blood that had the ability to interact with the toxic protein in a double diffusion reaction in the gel. In a 1:64 dilution the antiserum had 1 precipitation line with the SEB preparation (1 mg/ml), which confirmed the homogeneity of the isolated preparation. SEB migrated from the anode to the cathode; that is, it had a positive charge (figure 6) [photograph not reproduced].

We studied the serum in a double diffusion reaction in gel together with sera and preparations of type A and C staphylococcal enterotoxins, and with culture filtrates of staphylococcal strains that produce type A, C, D, and E enterotoxins and the Wood-46 type, which does not form an enterotoxin. We showed that precipitation takes place only with SEB and with culture filtrates of strains that produce type B enterotoxin. We observed a negative reaction with preparations of other types of staphylococcal enterotoxins and with culture filtrates of strains that form other types of enterotoxins and that do not form enterotoxins.

The type B staphylococcal anti-enterotoxin serum that we obtained is immunologically identical to the SEB serum produced by the "Serva" company (Figure 6) [photograph not reproduced].

Conclusions

1. A preparation of type B staphylococcal enterotoxin was obtained by successive performance of the following operations: adsorption from the culture fluid on amberlite, elution, isoelectric focusing in a system of ampholines, and gel filtration in a column with Sephadex G-25.

2. The enterotoxin is a thermostable protein with a molecular weight of 28,000, and an absorption maximum at 278 nm, pI 8.6. The minimum enterotoxin dose for cats is 3-5 μg per kg of body weight. The enterotoxin's biological activity disappears when acted upon by 6 M urea.

3. We obtained a monospecific anti-enterotoxin serum for type B staphylococcal enterotoxin with a titer of precipitating antibodies of 1:64. The serum is identical to the type B staphylococcal anti-enterotoxin serum produced by the "Serva" company.

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ISOLATION OF LEGIONELLA PNEUMOPHILA STRAINS FOR FIRST TIME IN THE USSR

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[Article by I. S. Tartakovskiy, S. V. Prozorovskiy, and V. L. Popov, Epidemiology and Microbiology Institute imeni N. F. Gamaleya of the USSR Academy of Medical Sciences, Moscow]

[Text] Over recent years more than 200 outbreaks and numerous sporadic cases of legionellesis have been reported [2, 9, 10]. The wide incidence of the disease, which, as a rule, takes the form of acute, severe pneumonia, often with a fatal outcome (15-20 percent mortality), demands that the specific epidemiological and clinical aspects of the disease be studied, along with the ecology and biology of its agent, and that laboratory diagnosis be developed.

In the diagnosis of legionellesis, serological research methods have been the most widespread. Using these methods, a positive diagnosis is made in more than 90 percent of the legionellesis cases [7]. There is significantly less success in isolating the agent directly from the patient. The agent is usually isolated by performing a lung biopsy on the patient; in rarer cases, it is isolated from the patient's pleural fluid, bronchial fluid, or blood [7, 8].

In the past in the USSR a whole series of serologically confirmed cases of legionellesis have been discovered; the incidence of legionellesis infection among acute pneumonias with a different etiology was 4-6 percent [1, 3]. The present study is devoted to isolating and describing the first strains of the legionellesis agent in the USSR.

Materials and methods. Cultures of the agent were isolated from the lung tissue of an 8-year old boy who was thought to have died from an acute respiratory infection and bilateral polysegmentary pneumonia in Tula; lung tissue from a 74-year old man in Voronezh who died from acute pneumonia with an undetermined etiology was also used. A section of lung tissue not larger than 1 cm^3 was ground in a mortar and suspended in 5 ml of a 0.05 M phosphate buffer (pH 7.2). Dilutions of the suspension (1:10 and 1:100) were placed in a charcoal-yeast agar without antibiotics and in a charcoal-yeast agar with vancomycin (1 microgram/ml) and with polymyxin B (5 micrograms/ml). We used an agar containing soy treated enzymatically by trypsin, Hottinger's agar, and a charcoal-yeast agar without L-cysteine and glandular pyrophosphate as control media. At the same time we introduced 1 ml of the original suspension

intra-abdominally into guinea pigs weighing between 250 and 300 g. We used a splenic suspension from the dead patients or from the diseased guinea pigs to infect 6-day old chicken embryos. The embryos died after 3-5 days; they were dissected and placed in culture media. The cultures were incubated at 36°C in a 5 percent CO_2 atmosphere. The culture dishes were observed for 10 days. The presumed colonies of legionnellae were placed in a charcoal-yeast agar and in control media.

We studied the morphological and culture aspects of the isolated strains using the methods described by Weaver [17]. We used APIZYM [15] to determine the biochemical characteristics of the strains. Hebert's method [11] was used for hydrolysis of the sodium hippurate. We determined the serological group of the isolated strains by means of indirect immunofluorescence using rabbit antisera for the strains Philadelphia I (serological group 1), Togus (serological group 2), Bloomington (serological group 3) and Los Angeles (serological group 4). Reference strains were obtained from the Center for Disease Control (Atlanta, USA). Antisera for the isolated strains and the reference strains were obtained according to the method described by Cherry et al. [6]. The titers of the antisera were no less than 1:1024. We analyzed the fatty acid spectrum of the strains by means of the high temperature transmethylation method using tetramethyl ammonium hydroxide. In order to determine the virulence of the strains, dilutions of the bacterial suspension were introduced into guinea pigs in 1 ml doses and into the yolk sac of chicken embryos in 0.3 ml doses.

In order to study the ultrastructural organization of the isolated cultures the colonies were separated from the agar and fixed with a mixture of formaldehyde, glutaric aldehyde, and picric acid prepared in a 0.2 M cacodylate buffer (pH 7.3-7.4) according to the method described by Ito and Karnovsky [12]. The fixative was applied for 1 hour at room temperature and then the preparations were placed in a refrigerator. After final fixation with 1 percent OsO_4, the preparations were treated with a 1 percent solution of uranylacetate in a maleate buffer with a pH of 6.0 for 1-2 hours, they were dehydrated in ethanol and rinsed in araldite M in flat dishes or capsules. In order to determine the surface acid mucopolysaccharides a 0.15 percent solution of ruthenium red stain was added to the fixatives according to the method described by Luft [13]. Ultrathin sections were obtained using an ultra-microtome LKB-8800 (Sweden); they were contrasted using a 5 percent aqueous solution of uranylacetate alone, or with the addition of lead citrate. They were examined using a JEM-100B electron microscope with accelerating voltage of 80 kilovolts and an objective diaphragm 50 μm in diameter.

Results and discussion. The Tula and Voronezh strains were isolated by both of the methods applied here. In the case of the Tula strain the colonies suspected of containing legionellae were cultured in a charcoal-yeast agar with vancomycin and polymyxin B 7-9 days after the lung tissue suspension was placed in the medium. In the medium without antibiotics, the abundant growth of extraneous microflora made it impossible to find colonies of the legionellesis agent. In the recultivation the suspected colonies grew in a charcoal-yeast agar after 3-4 days and did not grow in the control media without L-cysteine and iron pyrophosphate. The other method included additional selection of the agent in the the body of guinea pigs that had been infected with the lung tissue suspension. The diseased guinea pigs were killed 4-6 days following the

contamination and a spleen tissue suspension was placed in a charcoal-yeast agar and 5-6 day old chicken embryos were contaminated with this suspension. Colonies of legionella-like microorganisms grew in 5-6 days. Smears from chicken embryos that died after 4-5 days were stained according to Jimenez' method and contained a pure culture of thin bacilli 2-10 micrometers in length that are typical of legionellae.

The etiological role of the Tula strain was confirmed by direct immunofluorescence of impressions of lung tissue (figure 1) and by indirect immunofluorescence of the patient's blood serum. The titer of antibodies in the serum, taken on the sixth day of illness, was 1:512 with the antigen L. pneumophila of serogroup 1, prepared at the Epidemiology and Microbiology Institute imeni N. F. Gamaleya, and with the antigen obtained from the Center for Disease Control. In isolating the Voronezh strain, there was no blood serum from the dead patient and direct immunofluorescence of the lung tissue had a negative result.

Table 1 contains the primary culture and biochemical characteristics of the isolated strains that coincide with the characteristics of the typical Philadelphia I strain. Serologically the strains are described in terms of the reaction of indirect immunofluorescence with homologous rabbit antisera and antisera for the typical strains of the 4 serogroups of L. pneumophila (table 2). The antisera for the Tula and Voronezh strains in a double radial immunodiffusion reaction in 1 percent agarose formed precipitation lines with soluble antigens of the homologous strain, with the type-specific antigen of the Philadelphia I strain, and with the group-specific antigen isolated from the Philadelphia I and Togus strains of serogroups I and II. The antiserum for the isolated strains did not precipitate with the type-specific antigen of serogroup II. Thus, the antigenic property of the Tula and Voronezh strains indicates that they belong to serogroup I.

The original LD_{50} of the isolated strains for guinea pigs was $6 \cdot 10^2$-$8.5 \cdot 10^2$ colony-forming units, but after 4 passages in an artificial nutrient medium the value of LD_{50} rose to $4 \cdot 10^6$-$5 \cdot 10^6$ colony-forming units. Subsequent passages of the strains in chicken embryos and the body of a guinea pig made it possible to increase their virulence by an order of 2-3 using a method described earlier [4]. The data correspond to the results obtained by McDade and Shepard [14], who showed that the virulence of newly isolated legionellae drops markedly after several passages in an artificial nutrient medium.

Two types of bacterial orientation were observed in ultrathin sections of the legionella colonies. The more common of the two was random arrangement of the cells; less often we found the bacteria lying in parallel rows, that could gradually bend, while still preserving the parallel orientation of the bacteria. We did not find any differences in the ultrastructure of the two strains; therefore the description of the ultrastructure is the same for both strains.

Table 1. Phenotypical characteristics of the isolated legionella strains

(1)	Шт. (2)	Шт. ров (3)	Шт. Ме (4)
1. Рост: (5)			
а) на угольно-дрожжевом агаре (6)	+	+	+
б) на агаре Мюллера — Хинтона (7)	+	+	+
в) на агаре с триптическим перевароом сои (8)	—	—	—
2. Гидролиз гиппурата Na (9)	+	+	+
3. Окраска: (10)			
а) по Граму (11)	—	—	—
б) по Гименезу (12)	+	+	+
4. Образование коричневого пигмента (13)	+	+	+
5. Основная жирная кислота (14)	16:0	.	16:0
6. Биохимические реакции: (15)			
(16) а) наличие оксидазы	+	+	+
(17) б) наличие каталазы	+	+	+
(18) в) наличие уреазы	—	—	—
г) разжижение желатины (19)	+	+	+
д) NO_3—NO_2 (20)	—	—	—
е) утилизация крахмала (21)	+	+	+
ж) ферментация углеводов (22)	—	—	—

Note: The symbol . indicates that the value was not determined.

Key:

1. Characteristic
2. Tula strain
3. Voronezh strain
4. Philadelphia I strain
5. Growth
6. In charcoal-yeast agar
7. In Müller-Hinton agar
8. In an agar with soy treated enzymatically with trypsin
9. Hydrolysis of sodium hippurate
10. Stain
11. Gram's
12. Jimenez
13. Formation of brown pigment
14. Main fatty acid
15. Biochemical reactions
16. Presence of oxidase
17. Presence of catalase
18. Presence of urease
19. Thinning of gelatin
20. NO_3-NO_2
21. Utilization of starch
22. Breakdown of carbohydrates

In the colonies it was possible to distinguish several types of cells that were characteristic in terms of their morphology (figure 2) [photograph not reproduced]: the first type was represented by small (0.2-0.3 x 0.3 x 0.4 μm), slightly oval-shaped bacteria with dense cytoplasm and, as a rule, convolutions in the cell wall; the second type was represented by larger (0.5-0.6 μm), round cells with fairly thin cytoplasm and a different nucleoid; the third group contained bacteria in the form of short bacilli measuring 0.2-0.3 x 0.7-1.3 μm with dense cytoplasm and convolutions in the cell wall; the fourth group contained short, thick bacilli (0.3-0.4 x 0.8-1.0 μm), usually barrel-shaped, broader in the middle section, with a well-defined nucleoid; the fifth group

consisted of elongated cells (up to 2 micrometers) that were similar to the types described above in terms of their dense cytoplasm. Evidently the type 1 and 2 bacteria were cross sections or transverse sections of types 3 and 4, respectively. In addition to this, spheroplast-like cells were found in the colonies that were about 1 μm in diameter, along with bacteria containing very thin, apparently degenerated cytoplasm; membrane rings whose contents were transparent to electrons; membrane fragments; and fibrillar and structureless material.

Table 2. Titers of antibodies of rabbit antisera for typical Legionella strains and the isolated Legionella strains in the indirect immunofluorescence reaction

Антиген (1)	(2) Антисыворотки к штаммам				
	Тула (3)	Воронеж (4)	Philadelphia 1 (серотип 1) (5)	Togus (серотип 2) (6)	Bloomington 2 (серотип 3) (7)
Тула (8)	1:4096	1:1024	1:2048	1:64	1:32
Воронеж (9)	1:1024	1:1024	1:1024	1:32	1:32
Philadelphia 1	1:512	1:1024	1:2048	1:64	1:64
Togus	1:16	1:16	1:32	1:1024	1:16
Bloomington 2	1:16	1:32	1:32	1:16	1:2048

Key:

1. Antigen
2. Antisera for the strains
3. Tula
4. Voronezh
5. (Serotype 1)
6. (Serotype 2)
7. (Serotype 2)
8. Tula
9. Voronezh

The bacteria had a clearly defined cell wall and a cytoplasmic membrane (figures 3-6) [photographs not reproduced]. The three-layered membrane of the cell wall was about 10 nm thick, and its inner layer had a brighter contrast than the outer wall (figure 5) [photograph not reproduced], apparently due to the adjacent peptide-glycan layer. The surface of the cell wall did not react to a ruthenium red stain (figure 6) [photograph not reproduced] and flakes of electron-dense material were adjacent to the surface only in places. The narrow periplasmic space was about 10 nm in width and had a density similar to that of the cytoplasm; it divided the cell wall from the cytoplasmic membrane which was about 7 nm thick. In several legionella cells 1-2 additional membranes were found in the cytoplasm under the cytoplasmic membrane; they lay parallel to the cytoplasmic membrane and were similar to that membrane in terms of thickness (figure 3) [photograph not reproduced]. Oval vacuoles, characteristic of legionella cells, were found in the cytoplasm; they were encompassed by a single-contour membrane about 3 nm thick and the contents were transparent to electrons. Some larger round vacuoles were seen in some cells and were encompassed by a three-layer membrane 6 nm thick; they were filled with fibrillar contents (figure 4) [photograph not reproduced]. Cell division took place with the central part of the cells stretching apart (figures 2 and 6) [not reproduced]. Thus, the ultrastructure of the two strains of this agent which were isolated in the USSR is typical of the structure of legionella described earlier [16].

The cultural, morphological, biochemical, and serological properties of the isolated strains, together with the organization of the ultrastructure confirm that they belong to serogroup 1 of L. pneumophila.

The isolation of the first strains of the legionellesis agent in cases of lethal pneumonia, together with the results of serological research, demonstrate the need for a detailed study of the incidence of legionellesis in cases of other respiratory illnesses in the USSR. It is necessary to consider the possibility of outbreaks of legionellesis infection, including outbreaks within hospitals, which are being reported with increasing frequency in various countries of the world [5]. The presence of the agent in water supply systems and numerous cases of isolating this agent in the water systems of air conditioners, showers, baths, and other plumbing, indicate the need to study these systems in our own country. Here one cannot exclude the possibility of isolating new types of legionellae in the USSR that are not among the 8 types already known. Over 90 percent of the legionellae isolated in the world are in serogroup 1 of L. pneumophila.

Conclusions.

1. For the first time in the USSR, strains of legionellesis agents were isolated from patients who died from pneumonia.

2. On the basis of their cultural, morphological, biochemical, and serological characteristics, both the isolated strains were classified with serogroup 1 of L. pneumophila.

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POSSIBLE RELATIONSHIP BETWEEN VIRULENCE OF PSEUDOMONAS AERUGINOSA AND SOME FEATURES OF THEIR ULTRASTRUCTURE

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 (manuscript received 15 Mar 83) pp 45-47

[Article by S. T. Dzyubak, Ivano-Frankovsk Medical Institute]

[Text] Electron microscopy has been used to study P. aeruginosa: the ultrastructure of typical P. aeruginosa cells [4, 11], the particular distribution of flagella [3], capsule polysaccharides [10], the variability of the microbe under the influence of antibacterial preparations [2, 7], and physical factors [5]. In the literature there is practically no information on patients with purulent infections and from the environment, taking into account the biological activity of the microbes. This research comprises the basis of the present work.

Materials and methods. We studied 9 strains of P. aeruginosa, 5 of which were isolated from surgical patients with inflammatory purulent processes (including the standard, highly toxic strain PA-103); and 4 of which were isolated from soil and water. The bacteria were identified using generally accepted tests: Gram's stain, mobility, formation of pyocyanin, thermophilic nature, oxidation of glucose under aerobic conditions, oxidase activity, oxidation of calcium gluconate, and thinning of gelatin. For the electron microscopic studies we used cultures in a stationary stage of growth (18-24 hours) in a meat and peptone broth and in a meat and peptone agar. In several cases we used an agar prepared in a tryptic soy extract.

After cultivation the bacterial cells were rinsed in a physiological solution of NaCl and fixed with 2 percent glutaric aldehyde or with a solution of osmium tetroxide. The precipitate was rinsed in a phosphate buffer with a pH of 7.3-7.6 and a suspension of the bacteria was prepared which was placed on a grid tray for study. In some cases whole cells were contrasted with 1 percent uranylacetate.

In order to obtain ultrathin sections of the precipitate after centrifugation, it was dehydrated by means of increasing concentrations of ethanol and then poured into epoxy resins. The sections were contrasted according to the method described by Reynolds [9]; they were examined using a JEMB-100B electron microscope with an instrument magnification of up to 80,000.

Results and discussion. In the electron micrographs the P. aeruginosa cells appeared as bacilli with rounded ends, 0.5-1 x 1.5-3 μm in size, and they often appeared to be lying in a parallel arrangement. In the virulent strains cells were often found which were 2-3 times longer than normal cells with the same thickness, and there were individual cells with an ellipsoidal form. When a contrast was made with uranylacetate penetrating the cells, bipolar, sometimes round segments appeared in these cells with a high electron density.

In the newly isolated virulent strains, unlike the avirulent strains, a mucuoid capsule was found on the surface of the cells. Two layers that were quite distinct were seen in the capsule: an inner, rigid layer bound tightly to the cell wall; and an outer, thinner, homogeneous layer (see figure A) [photograph not reproduced].

It is characteristic that on the surface of the outer layer there were many small round formations. It is possible that these formations have adhesive properties that allow the bacteria to attach themselves to several substrates.

This type of mucoid capsule was seen particularly clearly in the bacteria that were grown in the nutrient media containing tryptic soy extract that were used to obtain the P. aeruginosa exotoxin [8]. In light of this, we can assume that this type of extracellular structure is a definite factor in the microbe's toxigenic activity. It has also been established that the prolonged presence of mucoid strains in dense nutrient media without recultivation allows them to maintain their viability. In similar cases the bacterial cells, usually a concentration of them (figure B) [not reproduced] were covered with a well-formed coating of mucus. In these cases the cells were smaller than ordinary cells; they were oval in form and had a high electron density. Surrounding the concentrations of cells one could clearly see an empty space separating the cells from the covering formation. Similar observations have been described by other authors [6] who isolated P. aeruginosa from the abdominal cavity of animals 5 hours after they were infected, and also in the study of whole Francisella tularensis cells [1]. It is entirely possible that the formation of the mucus layer on the surface of the cells can occur both in vivo and in vitro.

In an electron microscopic study of ultrathin sections, a general principle of organization of P. aeruginosa cells was discovered and differences were established in the structure of virulent and avirulent strains. The cells of avirulent strains of P. aeruginosa (figure C) [not reproduced] had a structure typical of the majority of gram-negative bacteria. The cell wall consists of a smooth, three-layered membrane and an inner layer with an average electron density. However, this similarity was not seen in all cells. Fairly often the outer membrane of the cell wall in the avirulent strain did not have the convolutions characteristic of gram-negative bacteria. The cytoplasmic membrane was not always separated from the cell wall. The cytoplasm had fine granules, a heightened electron density, and vacuoles. The nucleoid often appeared in the form of two clearly differentiated osmiophobic zones full of fine osmiophilic fibrils.

In an examination of cells of virulent strains, we found that their ultrastructure is quite different from that of cells of avirulent strains

(figures D and E) [not reproduced]. The cell wall had an indistinct contour structure, in various places it was separated from the cytoplasmic membrane and it had a high electron density. In some cells the cell wall and cytoplasmic membrane could not be seen due to lysis. Many osmiophilic granular particles were seen in the cytoplasm which varied in terms of their size and form and were located close to the cytoplasmic membrane and the poles of the cell. It is likely that these granular components are involved in the cell's toxin formation function.

One can assume that the appearance of the granular formations creates the conditions necessary for synthesis of the exotoxin and for transporting it into the environment. This assumption is based on the fact that these granules were found in cells of the highly toxic PA-103 strain, which are known absolutely to produce the exotoxin.

The cells of the virulent strains also differed from those of the avirulent strains in terms of the nucleoid arrangement. It is often arranged in a disordered way in the form of osmiophobic material.

Bubbles were also seen in the cytoplasm.

Thus, the research done here allowed us to reveal a number of morphological peculiarities in the subcellular structure of virulent and avirulent strains of P. aeruginosa, which indicate the high degree of polymorphism of that microbe, which is a factor of the nutrient medium.

It is possible that one of the reasons for the resistance of P. aeruginosa to the body's defense responses and to conditions of external influences is the formation of the mucous capsule.

Conclusions

1. Cells of the virulent strains of P. aeruginosa differ from cells of avirulent strains in that they have a mucoid capsule, there are variations in their size, and they form electron-dense structures at their poles.

2. Cells of virulent strains contain a significant number of granular cytoplasmic components and the cell wall has a less contoured structure than that found in avirulent strains.

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ENTEROTOXIC PROPERTIES OF SALMONELLAE AND NEUROTOXINS ISOLATED FROM THESE ORGANISMS

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[Article by N. B. Kamzolkina, Z. N. Yermolenko, and I. N. Kamenskaya, Epidemiology and Microbiology Scientific Research Institute imeni G. N. Gabrichevskiy, under the RSFSR Ministry of Health, Moscow]

[Text] The enterotoxic effect of salmonellae was first observed by Taylor and Wilkins in 1961 [10]. For a long time the numerous efforts to tie the entertoxic activity of salmonellae to their ability to produce enterotoxin met with failure [4]. Only in 1975 did Koupal and Deibel succeed in observing enterotoxic activity of a concentrated culture fluid after growing several types of salmonellae. Later on [8, 9] these investigators used ultrafiltration of the suprasedimentary culture fluid with subsequent chromatography in Sephadex G-100 and DEAE cellulose to obtain an enterotoxin that had been purified 50 times.

Materials and methods. This paper presents the results of a study of the enterotoxic properties of salmonellae using an isolated loop of a rabbit's small intestine as a model, and of the neurotoxins obtained from the salmonellae according to the method described by Mesrobeanu et al. [7]. We determined the enterotoxicity of 51 strains of Salmonella typhimurium; the contents of intestinal loops of rabbits who responded positively to the introduction of live cultures of these salmonellae; 8 neurotoxin preparations obtained from 5 strains of S. typhimurium of varying origin; 1 strain of S. enteritidis, obtained from a dead mouse; and 2 strains of S. typhi: ty 2-4446 and 66, obtained from a patient.

The strains of S. typhimurium were isolated from patients suffering from salmonellosis in Moscow, Moscow Oblast, and Derbent in the Dagestan ASSR. We also used strains of S. typhimurium isolated from the environment, from birds, and museum strains (811 and 669).

We usually studied up to 10-11 cultures in one rabbit; 1 billion microbial cells per 1 ml were introduced into the lumen of an intestinal loop; this quantity was determined according to the standard developed at the State Medical Biological Preparations Control Institute imeni L. A. Tarasevich. The rabbits were killed on the day following the operation; when the intestinal

loops were filled with exudate, we measured their length in centimeters and determined the amount of fluid in milliliters. We determined the enterotoxic properties of the cultures and toxins as the ratio between the volume of fluid in the segment and the segment's length (the enterotoxic index). A positive reaction was judged to be a index above 0.5; a ratio between 0.4-0.5 was considered a weak reaction; a ratio below 0.4 was considered a negative reaction.

At the same time, the contents of several loops were dissolved in a physiological solution of NaCl and were placed in dishes containing Levin's medium in order to evaluate the degree of multiplication of salmonellae in the lumen of the intestinal loops. In the absence of exudate 1 ml of a sterile physiological solution of NaCl was injected into the segment and after thorough concentration, the fluid was removed through a puncture in the intestinal wall so it could be used in a culture.

In a number of experiments we used rabbits that had been immunized beforehand with a suspension of salmonellae of various serotypes that had been killed by boiling. The immunization cycle consisted of 3 intravenous injections given at 5 day intervals. Doses of 10, 50, and 100 million microbial cells were used for the immunization. Rabbit No 242 was an exception: it was immunized with S. düsseldorf in doses of 500 million, 1 billion, and 1.5 billion microbial cells. The immunized rabbits were operated on 2 months after the end of the vaccination. The contents of the intestinal loops in which the exudate accumulated as a result of the introduction of live cultures, after centrifugation and separation from the precipitate, was decontaminated by adding several small quantities of chloroform; the material was kept in chloroform for 1-3 days under a rubber stopper. Prior to lyophilization the fluid was separated from the chloroform and was tested for sterility by placing it in a meat and peptone agar, a casein and sugar fluid medium, and Saburo and Kitt-Tarocci's medium. The freeze-dried contents of the intestinal loops were dissolved in a NaCl physiological solution in a 5:1 concentration and 1-3 ml of the solution were introduced into the rabbits. In the case of a positive reaction control cultures were made from the rabbits' intestinal contents in order to eliminate conclusively the possibility of isolating salmonellae that were not killed by the chloroform vapors and that were not sifted out when the sterility of the intestinal contents was being examined.

When we studied the enterotoxicity of the neurotoxin preparations, we used quantities of 0.8, 1.0, 1.5, and 3.0 ml.

Results and discussion. Tables 1 and 2 show that in nonimmune rabbits and rabbits immunized by salmonellae of heterologous serotypes, the accumulation of fluid in response to the introduction of live cultures of S. typhimurium occurred only in the upper sections of the small intestine. The museum strain 669, taken as a control, elicited an abundant exudation of serous fluid in isolated loops of the proximal section of the rabbit's small intestine (rabbits No 175 and 361); a similar reaction was not seen in the distal sections of the small intestines (rabbits No 376 and 233) or it was very weak (rabbits No 253 and 242). Depending on the position of the loop (the calculations were made from the proximal to the distal part of the intestine), the same culture of S. typhimurium behaved differently in tests on different rabbits. For example,

cultures 118, 198, and 307 in loops 2, 3, and 4 in rabbit No 376 produced positive reactions; while in loops 11, 10, and 9 in rabbit number 361 the reactions were negative.

Table 1. Enterotoxic activity and intra-intestinal reproduction capability of S. typhimurium (unimmunized rabbits)

№ кролика (1)	№ кишечной петли (2)	№ штамма (3)	Происхождение штамма (4)	Объем см/длина* (5)	Число бактерий (млрд. мк в 1 мл) (6)	№ кролика (1)	№ кишечной петли (2)	№ штамма (3)	Происхождение штамма (4)	Объем см* (5)	Число бактерий (млрд. мк в 1 мл) (6)
517	1	146	в/б (7)	0,78	4 983	175**	1	669	м (8)	1	310
	2	226	»	отр (9)	7 140		2	170	с/с (10)	отр (9)	3626
	3	71	»	»	60		3	169	(11)	»	
	4	163	»	»	4 258		4	4	в/ср (12)	»	
	5	181	»	»	1 570		5	18	в/ (7)	»	
	6	холерогеи (контроль) (13)		0,4	.		6	71	»	»	159
							7	75	с/	»	9,8
							8	35	» (10)	»	152
							9	209	в/б	»	
							10	31	» (7)	»	
376	1	28	п (14)	1,8	12 000	361	1	28	п (14)	0,4	5 500
	2	118	в/б	0,6	19 200		2	669	м (8)	1,2	2 383
	3	198	» (7)	0,65	19 000		3	490	с/с (10)	0,7	41 287
	4	307	»	1,7	17 000		4	482	в/б	отр (9)	
	5	425	»	0,6	.		5	809	» (7)	»	
	6	811	» (8)	0,4	.		6	886	»	»	
	7	86	в/б	отр	.		7	811	м (8)	»	
	8	809	» (7)	» (9)	.		8	425	в/б	»	
	9	482	»	»	.		9	307	» (7)	»	
	10	490	с/с (10)	»	.		10	198	»	»	
	11	669	м (8)	»	.		11	118	»	»	

Note: The symbol . indicates that a culture was not taken from the intestinal loop

*--enterotoxic index **--Rabbit No 175 had coccidiosis

Key:

1. Rabbit number
2. Intestinal loop number
3. Strain number
4. Origin of strain
5. Volume/length
6. Number of bacteria (billions of microbial cells in 1 ml)
7. Nosocomial salmonellosis
8. Museum
9. Negative
10. Sporadic salmonellosis
11. Contamination by contact
12. External environment
13. Cholerogen (control)
14. Bird

As tables 1 and 2 show, the response of a rabbit's small intestine to live salmonella cultures depended to a great extent on the sensitivity of the animal itself. In 3 rabbits (Nos 247, 226, and 222), immunized with vaccines from the same type of cultures (see table 2), the proximal segments of the small intestine loop did not respond at all to the enterotoxic activity of the salmonellae; only the distal intestinal loops reacted.

Table 2. Enterotoxic activity and intra-intestinal multiplication ability of live cultures of 5 types of S. typhimurium (immunized rabbits)

№ кролика, чем привит (1)	(2) № кишечной петли	(3) № штамма	(4) Происхождение штамма	(5) Объем/длина	(6) Число бактерий, млрд. мк в 1 мл содержимого петли кишечника
253 группа D S. typhi (O-901) (7)	1	124	с/с (9)	0,7	5 466
	2	141	в/б (12)	0,9	3 808
	3	145	»	0,5	4 641
	4	154	»	отр (11)	3 243
	5	155	»	»	.
	6	157	с/с (9)	»	.
	7	162	в/б (12)	»	.
	8	164	»	»	.
	9	166	с/с (9)	»	.
	10	669	м (10)	0,5	9 324
233 группа D S. typhi (O-901) (7)	1	71	в/б (12)	1,5	4 015
	2	75	с/с (9)	1,5	9 800
	3	18	в/б (12)	0,6	9 278
	4	169	к (14)	0,4	11 681
	5	31	в/б (12)	0,8	4 454
	6	170	с/с (9)	1,3	5 278
	7	35	»	1,3	1 774
	8	4	в/ср (15)	отр (11)	.
	9	209	в б (12)	»	.
	10	669	м (10)	»	.
242 — группа C_2 S dusseldorf (17) (8)	1	40	п (16)	1,2	740
	2	346	в/ср	0,8	614
	3	347	к (15)	отр (11)	.
	4	355	»	»	6 240
	5	178	п (17)	»	.
	6	877	»	»	.
	7	878	»	»	.
	8	83	»	»	.
	9	688	»	»	.
	10	669	м (10)	0,57	278

№ кролика, чем привит (1)	(2) № кишечной петли	(3) № штамма	(4) Происхождение штамма	(5) Объем/длина	(6) Число бактерий, млрд. мк в 1 мл
226 — группа B S. typhimurium (13)	1	669	м (10)	отр (11)	941
	2	124	с/с (9)	»	.
	3	141	в/б	»	.
	4	145	» (12)	»	518
	5	154	»	»	.
	6	155	»	»	.
	7	157	с/с (9)	»	.
	8	162	в/б (12)	»	.
	9	164	»	»	.
	10	166	с/с (9)	»	.
	11	669	м (10)	1,3	981
222 — группа B (S. typhimurium) (13)	1	669	м (10)	отр (11)	.
	2	71	в/б (12)	»	.
	3	75	с/с (9)	отр (11)	.
	4	18	в/б (12)	1,0	948
	5	169	к (14)	отр (11)	.
	6	31	в/б (12)	»	.
	7	170	с/с (9)	»	.
	8	35	»	»	.
	9	4	в/ср (15)	»	.
	10	209	в/б (12)	1,0	284
	11	669	м (10)	1,1	609
247-группа B (S. typhimurium) (13)	1	669	м (10)	отр (11)	.
	2	40	п (16)	»	.
	3	346	в/ср	»	.
	4	347	» (15)	»	.
	5	355	п (17)	»	.
	6	178	п (17)	»	.
	7	877	»	»	.
	8	878	»	1,2	1278
	9	83	»	отр (11)	.
	10	688	»	1,5	988
	11	669	м (10)	1,5	154

Key:

1. Rabbit number, type of inoculation
2. Intestinal loop number
3. Strain number
4. Origin of strain
5. Volume/length (enterotoxic index)
6. Number of bacteria (billions of microbial cells in 1 ml of intestinal loop contents)
7. Group D
8. Group C_2
9. Sporadic salmonellosis

10. Museum
11. Negative
12. Nosocomial salmonellosis
13. Group B
14. Contamination by contact
15. External environment
16. Nasopharynx
17. Bird

This situation was confirmed especially graphically in the instance of the control strain 669, which was introduced into each rabbit at the first and last intestinal loop.

Judging from the data in tables 1 and 2, there was no connection between the enterotoxic capacity of the strains of S. tymphimurium and their origin. However, additional research is needed to make a conclusive judgment.

The results of the cultures indicate that salmonellae are capable of multiplying in the intestinal loop contents, and sometimes very intensively (see tables 1 and 2). It should be pointed out that the salmonellae multiplied even when they showed no enterotoxic activity (see table 1, rabbits No 175 and 517). Salmonellae also multiplied in the intestinal loops of rabbits that had been immunized both specifically and nonspecifically; they also multiplied both when they demonstrated an enterotoxic capacity and when they did not (see table 2).

Since the data on the reproduction rate of various cultures of S. typhimurium differed significantly among various rabbits, we calculated the geometric mean of the reproduction rates of salmonellae for the various groups of rabbits. Table 3 shows that a statistically significant difference in the reproduction rate of salmonellae from intestinal loops that reacted and did not react to the enterotoxin was seen only in unimmunized rabbits.

Table 3. Geometric mean reproduction rate of S. typhimurium from rabbits' intestinal loops (billion microbial cells in 1 ml)

(1) Энтеротоксичность	(2) Неиммунные кролики	Кролики, иммунизированные сальмонеллами (3)	
		гетерологичными (4)	гомологичными (5)
(6) Обнаружена	7343 ± 3.1*	3323 ± 0.24*	490.2 ± 1.97*
(7) Не обнаружена	471.9 ± 0.86*	4498 ± 1.78*	698.1 ± 33.52*

*Significant intervals were calculated for a 95 percent probability.

Key:

1. Enterotoxicity
2. Nonimmune rabbits
3. Rabbits immunized with salmonellae
4. Heterologous salmonellae
5. Homologous salmonellae
6. Observed
7. Not observed

In rabbits immunized with a vaccine of S. typhimurium, homologous microbes multiplied in intestinal loops that reacted positively to the enterotoxin to a

degree significantly lower than in intestinal segments in nonimmune rabbits or in rabbits immunized with a vaccine of heterologous strains. This difference was statistically significant. A statistically significant difference was observed in the reproduction rate of cultures from the intestinal loops of rabbits immunized with vaccines of homologous or heterologous strains and the reproduction rate in unimmunized rabbits in those cases in which the salmonellae proved to be enterotoxic.

If the strains did not cause an accumulation of fluid in isolated intestinal loops, no difference was seen in the reproduction rate of salmonellae from nonimmune rabbits and rabbits immunized by homologous salmonellae.

No correspondence was seen between the quantity of cultured salmonellae and the enterotoxicity of different strains in tests on the same rabbit. There was no correlation between the extent of the salmonella's intra-intestinal multiplication and its origin.

The sterilized contents of a rabbit's intestinal loops that showed a positive response to enterotoxic cultures of salmonellae were concentrated 5 times and introduced into other rabbits; a positive response was obtained when at least 2.5-3 ml were introduced.

Neurotoxin preparations obtained from 5 strains of S. typhimurium were tested on 4 rabbits; it was established that all the neurotoxin preparations had enterotoxic activity when introduced into the first 4-5 segments of the intestine.

Of the other neurotoxin preparations, only the neurotoxin from the S. typhi ty2 strain elicited a positive reaction in an isolated loop of the small intestine. Here hemorrhagic changes were seen in segments of the mucosa and the exudate contained some blood. A negative result was obtained when 10 mg/ml of the O-antigen from S. typhi 5501 strain was tested on one rabbit (in loop 5) and when 20 mg of the Vi-antigen was tested (loop 6).

Thus, the results of this research showed that the enterotoxicity of live cultures of S. typhimurium is tied to the salmonella's ability to produce enterotoxin, which can be extracted according to the method described by Mesrobeanu et al. [7].

Earlier we showed that neurotoxins extracted by this method from Escherichia coli [1], Shigella dysenteriae 1 and S. sonnei [2], have enterotoxic properties. In the present study we established this for S. typhimurium and S. typhi.

Some German authors [6] have referred to a similarity between the salmonella enterotoxin and the neurotoxin obtained from these microbes using the method described by Mesrobeanu et al. [7], but there is a lack of experimental data on this question. Evidently, the extraction of lipids from the cell wall of enterobacteria by chloroform [5] promotes the release of enterotoxin from these cells.

The method of using isolated segments for determining the enterotoxic activity of salmonellae cultures as well as their toxins has many defects. Besides the

well known significant differences in the rabbits' individual sensitivity, it turns out that only the upper sections of the small intestine react to the salmonella enterotoxin.

For the first time it has been established that parenteral immunization with a corpuscular vaccine of S. typhimurium causes the proximal sections of the rabbit small intestine to lose their sensitivity, and apparently, it sensitizes the distal sections of the small intestine to the effect of a homologous enterotoxin. These facts need to be confirmed through additional research.

The enterotoxic activity of several strains of S. typhi became known in 1980 [11]. Earlier we showed [1] that the neurotoxin from S. typhi is a substance which, in addition to the O-, Vi- and H-antigens, contains thermolabile intergeneric antigens that are common to pp. Salmonella, Shilgella, and Escherichia. In this study we established that salmonella neurotoxins have an enterotoxic action. This fact deserves serious study. There is no doubt that additional experiments are needed to prove conclusively that the neurotoxins' enterotoxic action does not depend on the O-, Vi-, and H-antigens.

Conclusions

1. The enterotoxicity of strains of S. typhimurium and S. typhi is a stable property, since museum cultures demonstrated a marked enterotoxicity.

2. All the strains of S. typhimurium studied multiplied in isolated loops of the rabbit small intestine, regardless of their origin and enterotoxic properties. Strains of S. typhimurium multiplied in the lumen of an isolated section of the small intestine in nonimmune rabbits more intensively in the presence of a marked exudative reaction than in the absence of one.

3. Neurotoxin preparations obtained according to the method described by Mesrobeanu et al. from cultures of S. typhimurium and S. typhi had enterotoxic effects.

4. In nonimmune rabbits and rabbits immunized parenterally with vaccines of salmonellae from serotypes not included in serogroup B, only the proximal section of the small intestine showed any sensitivity to the enterotoxic action of live cultures of S. typhimurium and their neurotoxin; in rabbits immunized with a vaccine of S. typhimurium, only the distal section showed any sensitivity; in the latter case there was a decline in the intensity of the multiplication of S. typhimurium in the lumen of the small intestine.

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PRECIPITATING ANTIGENS OF CAUSATIVE AGENTS OF PLAGUE, GLANDERS, AND MELIODOSIS WITH AQUEOUS SALINE EXTRACTS OF DIFFERENT PLANT SPECIES

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11 1983 (manuscript received 26 Oct 82) pp 57-58

[Article by A. M. Loktionov, I. N. Gaitova, and V. M. Samygin, Volgograd Antiplague Scientific Research Institute]

[Text] Numerous research studies have confirmed that there are various agglutinins and precipitins in aqueous saline extracts of seeds and other parts of plants; they are called phytohemagglutinins and lectins [1, 6]. For example, extracts from the leaves of the ornamental plant Cotyledon scheidecheri agglutinated cultures of Salmonella typhi and Vibrio cholerae, and they also precipitated proteins from horse and frog serum [2].

Many authors have described the precipitation of proteins, polysaccharides, and glycoproteins caused by lectins.

Repeated efforts to find lectins that interact specifically with bacterial antigens have met with little success. Relatively recently two papers appeared on the possibility of selective diagnosis of streptococci from the oral cavity (Streptococcus mutans) using lectins from rutabagas and of the gonorrhea agent using lectins from the wheat germ [4, 5].

In the available literature we did not find any information on the interaction between aqueous saline extracts of plants and antigens of agents of particularly dangerous diseases.

The aim of the present work was to test aqueous saline extracts from seeds of various types of plants with antigens of agents of plague, glanders, and melioidosis in an immunodiffusion reaction in gel.

Materials and methods. Our research materials included seeds from various types of plants; antigens of plague agents (fractions 1 and 2, obtained according to the method described by Baker); cultures of glanders agents (Pseudomonas mallei 10230) and melioidosis agents (P. pseudomallei 59361) in a concentration of $2\text{-}4 \cdot 10^{10}$ microbial cells per ml; these agents had been killed with 0.5 percent formalin; agar (Difco); normal rabbit serum obtained at the Volgograd Antiplague Scientific Research Institute; phytohemagglutinins and

bovine serum produced at the Olayne plant; and concanavalin A from the "Serva" company.

To obtain the aqueous saline extracts we poured seeds that had been ground up in a mortar into a 0.85 percent NaCl solution buffered with a 10 percent 0.01 M phosphate buffer solution (pH 7.2) in a weight ratio of 1:5-1:10. The material was extracted for 18 hours at room temperature and was clarified by centrifugation. The immunodiffusion reaction was set up according to the generally accepted method and the results were recorded after 18 hours of incubation at room temperature.

Results and discussion. Of the 55 aqueous saline extracts tested, 12 had positive precipitation reactions with the group of antigens used here (see table). As a rule, the extracts had an immunodiffusion reaction with several of the antigens. Extracts from date pits had heightened activity in the reaction with fractions 1 and 2 of the plague microbe; corn seed extracts had increased activity with the melioidosis agent. Their titers were 1:2, 1:4, 1:8, respectively. Extracts from buckwheat, date pits, and apple seeds did not have a positive immunodiffusion reaction with antigens of P. mallei. The overwhelming majority of extracts reacted in the immunodiffusion reaction with proteins from normal rabbit serum. The commercial lectins phytohemagglutinin and concanavalin A did not react with the selected group of antigens, but they did precipitate the normal rabbit serum and the bovine serum albumin. An example of the positive immunodifffusion reaction of the aqueous saline extract of date pits is presented in the accompanying photograph [photograph not reproduced].

The immunodiffusion reaction of extracts of various types of plants and antigens of plague, glanders, and melioidosis agents

(1) № п/п	(2) Экстракты семян	(3) Антигены				
		Y. pestis		P. mallei 10230	F. pseudomallei 59361	(6) НКС
		Ф I (4)	Ф II (5)			
1	Гречиха (7)	+	+	—	+	+
2	Финики (8)	1:2	1:4	—	+	+
3	Миндаль (9)	+	+	0	+	+
4	Чечевица (10)	+	+	+	+	+
5	Рапс (11)	+	+	+	+	0
6	Кукуруза (12)	+	+	+	1:8	+
7	Виноград (13)	1:32	1:32	1:32	1:32	+
8	Яблоки (14)	+	+	—	+	+
9	Персик (15)	+	+	+	+	0
10	Тыква (16)	+	+	+	+	0
11	Ревень (17)	+	+	+	+	+
12	Желуди (18)	+	+	+	—	+
13	ФГА (19)	—	—	—	—	+
14	Кон А (20)	—	—	—	—	+

Note: + represents a positive reaction; - represents a negative reaction; 1:32 is the reaction titer; 0 indicates that the tests were not done.

Key:

1. Number (in order)
2. Type of seed extract
3. Antigens
4. Fraction 1
5. Fraction 2
6. Normal rabbit serum
7. Buckwheat
8. Dates
9. Almonds

10. Lentil

11. Rape
12. Corn
13. Grapes
14. Apples
15. Peach
16. Pumpkin
17. Rhubarb
18. Acorns
19. Phytohemagglutinin
20. Concanavalin A

The extracts of rhubarb and grape seeds maintained their original activity after being heated at 70°C for 15 minutes; the same was true for the acorn extract which was heated at 100°C for 60 minutes. When the rhubarb seed and acorn extracts underwent dialysis through a cellophane membrane and tap water for 24 hours, they completely lost their precipitating activity. This can be explained by the fact that the active principle has a sufficiently low molecular weight [to $3 \cdot 10^4$-$4 \cdot 10^4$ atomic units) and is removed during dialysis.

Thermostabile lectins have been described in the literature [1-3]. Still, for conclusive proof of the lectin nature of the precipitation activity that we observed in the interaction of aqueous saline extracts of plants with bacterial antigens, it is necessary to prove in each case that the active principle is protein and certain radicals of carbohydrate molecules participate in the reaction.

Conclusion

In a study of the interaction of aqueous saline extracts of 55 types of plants in an immunodiffusion reaction in gel with antigens of plague, glanders, and melioidosis agents, the aqueous saline extracts of seeds from 12 types of plants had positive reactions. A number of extracts reacted positively with normal rabbit serum and bovine serum albumin.

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DIAGNOSTIC TEST SYSTEM FOR QUANTITATIVE DETERMINATION OF SHIGELLA ANTIGEN IN PATIENTS' BLOOD BY ELISA

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII, No 11, 1983 (manuscript received 13 Dec 82) pp 64-67

[Article by S. V. Shabalina, E. G. Abdurakhmanova, and V. P. Timina, Central Epidemiology Scientific Research Institute of the USSR Ministry of Health, Moscow]

[Text] In the diagnosis of acute dysentery, methods for determining specific shigella antigens in biological substrates are of particular interest. The primary advantage of these methods over bacteriological methods is the possibility of rapidly obtaining results in the early stages of the disease. In contemporary clinical immunology, a number of methods are used to identify specific antigens, including the complement fixation reaction, the indirect hemagglutination reaction, the aggregate hemagglutination reaction, the radioimmunology method, and others [1, 7]. These reactions, however, have significant shortcomings that limit their application. In particular, the indirect hemagglutination reaction and the aggregate hemagglutination reaction make it possible to give basically a semi-quantitative evaluation of the results, and the radioimmunology method requires complicated equipment and a large number of special reagents. Therefore, it is very important that we search for and apply new and better diagnostic tests that will make it possible to make diagnoses in the early stages of the disease.

The results of research that is being carried out intensively abroad and in our country have shown that the immuno-enzymatic analysis is very promising in this regard [2-5]. This test is characterized by a high degree of sensitivity, rapid set-up, and the possibility of making a quantitative analysis of the reaction results, which is very important for an objective evaluation of the activity of the pathological process.

The aim of the present study was to develop the optimal parameters for a diagnostic test system for determining the antigens of S. sonnei and S. flexneri in the blood of patients suffering from acute dysentery using the immuno-enzymatic analysis method.

Materials and methods. We used microtitration polystyrene plates from the "Cooke Microtiter" company as the solid foundation for joining the antibodies. We used S. sonnei and S. flexneri antigens obtained by means of the water and

phenol extraction method developed by Vestfal' at the Allergy and Immunology Laboratory of the Central Epidemiology Scientific Research Institute.

As the source of specific antibodies we used rabbit sera immune to S. sonnei and S. flexneri prepared at the Leningrad Epidemiology and Microbiology Scientific Research Institute imeni L. Pasteur. The globulin fractions of the dysentery sera were obtained according to Polson's method which involves two-fold sedimentation using a 20 percent solution of polyethylene glycol with a molecular weight of 6000.

The enzymatic conjugation of the gamma-globulin fractions was carried out according to the method described by Nakane [8], which is based on periodate oxidation of the inactive polysaccharide part of the peroxidase molecule; as a result of this, the aldehyde groups that are formed in the peroxidase react with the amino groups of the antibody molecules. We used a peroxidase from the "Serva" company, R_z=3.0 (R_z is an indicator of the enzyme's degree of purity; it is the ratio between the results of measuring the absorption of a peroxidase solution at two different wave lengths--OD_{403} and OD_{280} [expansion unknown]. The optimal value is 3.0.) We added 12 mg of peroxidase per 10 mg of protein.

To prepare the substrate mixture we dissolved 80 mg of 5-aminosalicylic acid at 70°C in 100 ml of distilled water and brought the pH of the resulting solution to 6.0 using a 0.1 N solution of NaOH. We added a working solution of H_2O_2 to the resulting mixture in a ratio of 10:1. The substrate was prepared right before it was used.

We used a variation of immuno-enzymatic analysis, the ELISA test (enzyme-linked immunosorbent assay). We evaluated the results of the reaction by the intensity of the staining of the substrate. The reaction was checked visually after keeping the plates at room temperature for 1 hour; an instrumental evaluation was made by measuring the extinction indicator on a "Dynatech" enzymometer. With the aim of making a direct evaluation of the absolute quantity of dysentery antigens we made a graph for each experiment showing the correlation between the extinction indicator and the quantity of antigens. In order to do this the antigen solution in each dish was titrated with a known concentration.

Results and discussion. As a result of this research, we developed the following optimal parameters for a diagnostic test system: a) the sensitizing doses of the antibodies; b) the pH buffer solutions for diluting the reagents; c) temperature and time conditions.

The optimal sensitizing doses of specific gamma globulins were determining by titrating them at various concentrations (from 5 to 200 mg/ml). Figure 1 presents the results of selecting the optimal sensitizing doses of specific gamma globulins in the form of extrapolation curves. The maximum sensitivity and the most stable results were obtained when we used gamma globulins for S. sonnei in a concentration of 25 µg/ml, and gamma globulins for S. flexneri in a concentration of 50 µg/ml.

Fig. 1

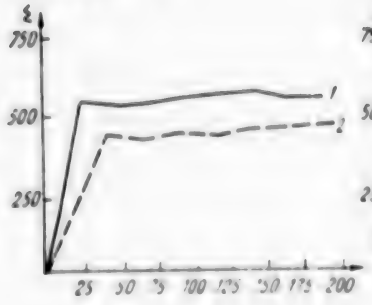

Fig. 2

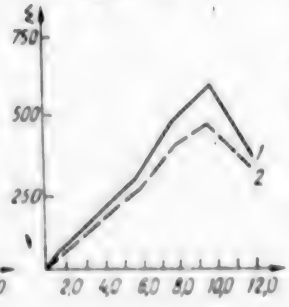

Fig. 3

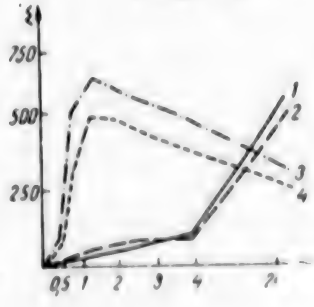

Figure 1. Selection of the optimal sensitizing doses of specific gamma globulins for antigens of S. sonnei (1) and S. flexneri (2). The horizontal axis represents the doses of gamma globulins (in μg/ml); the vertical axis represents the extinction indicators.

Figure 2. The effect of the pH of the buffer solutions on the serological activity of specific gamma globulins for S. sonnei (1) and S. flexneri (2). The horizontal axis represents the pH of the buffer solutions; the vertical axis represents the extinction indicators.

Figure 3. The effect of temperature and time of incubation on the serological activity of specific gamma globulins for S. sonnei (1) and S. flexneri (2) at 4°C; of Sonne's antigen (3) and Flexner's antigen (4) at 37°C. The horizontal axis represents the time of incubation (in hours); the vertical axis represents the extinction indicators.

To determine the effect of the pH of the buffer solutions on the serological activity of the antibodies we used potassium phosphate and carbonate buffer solutions with pH ranging from 2.0 to 12.0. Figure 2 presents the results of correlating the serological activity of the specific gamma globulins and the buffer solutions. The maximum serological activity of the gamma globulins was observed when 2 buffer solutions were used: a potassium phosphate buffer with a pH of 7.4 and a compensating solution with a pH of 9.6.

A comparative analysis of the reaction under different temperature conditions (37° and 4°C) and various incubation periods for the antibodies and antigens (0.5, 1, 2, 3, 4, and 24 hours), showed that the most rational conditions were incubation of polystyrene dishes with gamma globulins for 24 hours at 4°C (see figure 3). The serological activity of the antigens (when patients' serum was added) was optimal when the incubation was for 1.5 hours at 37°.

Thus, the tests on a number of parameters of the diagnostic test system allow us to suggest some optimal conditions for setting up a reaction to determine S. sonnei and S. flexneri antigens in patients' blood using immuno-enzymatic analysis: the sensitizing dose of Sonne's gamma globulin is 25 μg/ml; of Flexner's gamma globulin, it is 50 μg/ml; the pH of the buffer solutions for sensitizing the antibodies is 7.4 and 9.6; the time and temperature of incubation for gamma globulins is 24 hours at 4°C and for antigens it is 1.5 hours at 37°.

This diagnostic test system made it possible to determine the S. sonnei and S. flexneri antigens with an accuracy of up to 10^{-3} µg/ml.

To test this method clinically, we studied 1207 samples of blood serum from 564 people, including 256 who were suffering from acute dysentery. The control group consisted of 54 patients with a bacteriologically confirmed diagnosis of salmonellosis and 154 healthy individuals. We compared the frequency and level of the shigella antigens that were found in those suffering from acute dysentery and in the healthy individuals. In the healthy group we found the S. sonnei antigen in 6 out of 154 people (4 percent) and the S. flexneri antigen in 8 of 154 (5 percent). Among the 54 people suffering from bacteriologically confirmed salmonellosis, the S. sonnei antigen was found in 5 people (9 percent); the S. flexneri antigen was found in 7 people (12 percent). Among the 296 people with acute Flexner's dysentery, the Flexner-specific antigen was found in 213 (72 percent); among 60 people suffering from acute Sonne's dysentery, the Sonne-specific antigen was found in 42 (70 percent).

The level of specific shigella antigens in healthy individuals did not exceed 12^{-3} µg/ml; in individuals suffering from salmonellosis, the S. sonnei antigen was found in quantities up to 32^{-3} µg/ml and the S. flexneri antigen was found in quantities up to 64^{-3} µgml; in people with acute dysentery these antigens were found in concentrations of up to 125 µg/ml of serum.

Thus, we found statistically significant differences in the incidence and level of specific dysentery antigens found in patients suffering from dysentery and in control individuals.

The results of the study show that the immuno-enzymatic analysis method can be used to establish the etiology of acute dysentery.

Conclusions

1. We developed the optimal parameters of a diagnostic test system for establishing the presence of S. sonnei and S. flexneri antigens in the blood of patients with acute dysentery.

2. Using this test system, we found statistically significant differences in the frequency and level at which specific antigens of dysentery agents are found in the blood of patients and healthy individuals.

3. We demonstrated the possibility of using the test in making a serological diagnosis of acute dysentery.

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SENSITIVITY OF DIFFERENT CELL POPULATIONS IN IMMUNE SYSTEM OF MICE TO TICK-BORNE ENCEPHALITIS VIRUS

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII, No 11, 1983 (manuscript received 24 Feb 83) pp 75-78

[Article by V. V. Vargin and B. F. Semenov, Poliomyelitis and Viral Encephalitides Institute of the USSR Academy of Medical Sciences, Moscow]

[Text] In recent decades a very important feature of the pathogenesis of viral infections has been discovered. It has been determined that many viruses can multiply in the cells of the immune system, which results in a nonspecific modulation of the immune response [4, 5].

Cells have been found in populations of mice B- and T-lymphocytes and monocytes that are accessible to the lymphocytic choriomeningitis virus [7]. The T-lymphocytes of a sensitive host can be infected by adenovirus [8], the herpes simplex virus, and the simian herpes virus [11, 12]. Many viruses multiply in macrophages [4, 5, 9].

We studied the sensitivity of various cell populations in the immune system of mice to the tick-borne encephalitis (TBE) virus in vivo.

Materials and methods. For the experiments we used BALB mice weighing 18-20 g, obtained from the breeding facility of the USSR Academy of Medical Sciences. The animals were infected in the abdomen with the Sofyin TBE strain using a dose of $3 \cdot 10^5$ BOE/0.3 ml [expansion unknown]. We then determined which cells in the spleen, thymus, and bone marrow were capable of forming infection centers.

To indentify these cells we used suspensions containing $2 \cdot 10^5$-$2 \cdot 10^7$ live cells per 1 ml. The erythrocytes were first removed from the suspensions by means of 0.83 percent NH_4Cl. We placed 0.2 ml of the suspension on a monolayer of a mixed culture of renal epithelium from a mature pig; the cultures were placed in a thermostatic chamber for 60 minutes at 37°C; then an agar covering with a neutral stain was placed in the flasks [14]. The cultures were incubated in the thermostatic chamber for 6-7 days, after which we determined the number of negative colonies (markers). The number of cells capable of forming infection centers was expressed as the difference between the number of infection centers in the flasks containing the cells being studied, and the number of cells in the cultures that had been inoculated with a fluid fraction (the suprasedimentary fluid) of the cell suspension.

The T-lymphocytes were removed by means of the immune cytolysis reaction; this reaction was set up using anti-theta serum and complement from a guinea pig. We obtained anti-theta serum by immunizing AKR mice with thymocytes from CBA mice [10]. The guinea pig serum which served as complement was first adsorbed by spleen cells from BALB/c mice for 30 minutes at 4°C. The cytolysis reaction was carried out at 37°C.

The cells infected with the virus were lysed by specific antibodies in the presence of complement [3]. Commercial equine gamma globulin from the Tomsk Vaccines and Sera Scientific Research Institute was used as the source of antibodies against the TBE virus. We used fresh serum from guinea pigs as complement.

In order to separate the macrophages, $3 \cdot 10^7$ cells, suspended in 3 ml of medium No 199 with 3 percent calf serum, were placed in Petri dishes 10 cm in diameter. After incubation for 2 hours at 37°C, the non-adhering cells were removed by rinsing; the cells remaining on the glass, which accounted for 5-7 percent of the original population, were removed with a spatula and used in later experiments.

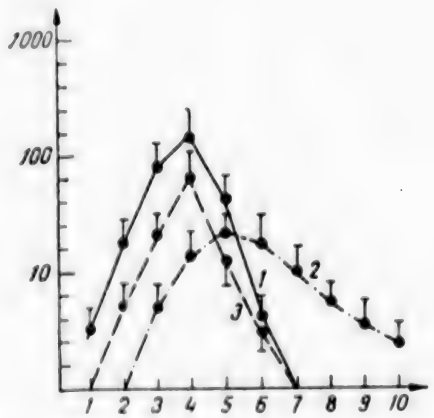
The geometric mean indicators of the number of cells capable of forming infection centers obtained in the experiments were analyzed using the Student's t-test [6].

Results and discussion. Preliminary experiments established that the TBE virus is capable of multiplying in the spleen, thymus, and bone marrow of mice. The data presented in the accompanying graph show that the TBE virus was found in splenocytes and bone marrow cells 24 hours after it was introduced into the abdomen. The TBE virus was found in thymocytes on the second day of the experiment.

The largest number of cells capable of forming infection centers was found in the spleen (80-200 per 10^6 cells). Fewer infected cells were found in the bone marrow (50-90 per 10^6 splenocytes) and in the thymus (20-40 per 10^6). The infection of immune system organs was short term. Seven days after the contamination no TBE virus was found in the splenocytes or in the bone marrow cells. The TBE virus remained in the thymocytes for a longer period of time (up to 10 days, when there paralysis developed and animals died).

The formation of infection centers in the culture of renal epithelium from a mature pig was not related to the presence of free virus or virus that had been adsorbed by cells in the cell suspensions being studied. The formation of negative colonies took place only when the immune system cells introduced into the test system were capable of synthesizing proteins. After the splenocytes were treated with cycloheximide, the number of marker cells in the culture of mature pig renal epithelium dropped to one-twenty-second of the quantity in the control (see table). These results permit us to suppose that there is active multiplication of the virus in the cells of the immune system. Also supporting this supposition are the results of experiments to determine the sensitivity of the splenocytes and the subpopulations isolated from them to the action of antibodies against the TBE virus in the presence of complement. The

development of immune cytolysis indicates the presence of specific antigen determinants on the cytoplasmic membranes of cells capable of forming infection centers, which appear in the process of the virus' replication. After the destruction of cells that carry viral antigens on their surface, there was a significant decrease in the number of cells capable of forming infection centers in the splenocyte culture.



The dynamics of the accumulation of cells capable of forming infection centers in the spleen, thymus, and bone marrow of BALB/c mice infected with TBE virus. 1--The results of identifying cells capable of forming infection centers among the splenocytes; 2--among the thymocytes; 3--among bone marrow cells. The horizontal axis represents the amount of time following the infection (in days); the vertical axis represents the number of cells capable of forming infection centers per 10^6 cells studied.

It has been established that an increase in splenocytes' functional activity led to a rise in the number of cells capable of forming infection centers that were found in the suspensions. For example, the original suspension of splenocytes from infected mice contained 161-198 cells capable of forming infection centers per 10^6 cells; after contact with the mitogen (phytohemagglutinin), this indicator rose to 216-258. Consequently, the intensity of the infection process in splenocytes depended on the level of the exchange processes, since the effect of phytohemagglutinin on the cells is tied primarily to a change in the synthesis of DNA [13].

The results of the research allow us to say that the TBE virus multiplies at least in two populations of spleen cells: in the population with characteristics of T-lymphocytes (the presence of the theta antigen and inability to adhere to a glass surface) and in the population with characteristics of macrophages (cells that adhere to a glass surface and that are without the theta antigen).

Thus, this research has expanded the list of viruses that are capable of multiplying in the T-lymphocytes of a sensitive host. The in vivo experiments confirmed reports based on in vitro experiments that showed that macrophages are a system that is accessible to the TBE virus [5]. We demonstrated a correlation between the sensitivity of these immune system cells to the TBE virus and their functional activity. In the culture of splenocytes stimulated

by phytohemagglutinin, there was a larger number of cells infected by the TBE virus than in populations of intact cells.

Characteristics of splenocytes that form infection centers

Исследуемые клетки (1)	Обработка (2)	Число инфекционных центров (на 10^6 клеток) (3)	
Спленоциты (4)	Не проводили (7)	178	$P=0,01$
	Циклогексимид* (8)	8	
	Анти-тета-сыворотка (АТС) (9)	180	$P<0,05$
	АТС + комплемент (С) (10)	94	
	Антитела к ВКЭ (АтВКЭ) (11)	123	$P=0,001$
	АтВКЭ + С (12)	5	
Фракция не прилипающих к стеклу спленоцитов (5)	Не проводили (7)	142	
	АТС (9)	138	$P<0,05$
	АТС + С (10)	78	
	АтВКЭ (11)	102	$P=0,001$
	АтВКЭ + С (12)	1	
Фракция прилипающих к стеклу спленоцитов (6)	Не проводили (7)	34	
	АТС (9)	36	$P>0,1$
	АТС + С (13)	39	
	АтВКЭ + С (12)	1	$P=0,001$
	АтВКЭ (11)	21	

*The cells were incubated in medium No 199 that contains cycloheximide in a concentration of 200 μg/ml.

Key:

1. Type of cell
2. Treatment
3. Number of infection centers (per 10^6 cells)
4. Splenocytes
5. Fraction of splenocytes not adhering to glass
6. Fraction of splenocytes adhering to glass
7. No treatment
8. Cycloheximide
9. Anti-theta serum
10. Anti-theta serum + complement
11. Antibodies against TBE virus
12. Antibodies against TBE virus + complement

The data on the ability of the TBE virus to multiply in cells with characteristics of T-lymphocytes and macrophages correspond to the facts described earlier concerning the nonspecific modulation of the immune response to unrelated antigens in experimental infection by TBE virus [1-3].

Theoretically, the consequences of viral infection of immune system cells can be varied: the infected cells can die; they can retain their viability but have a loss of functions tied to the reaction to the antigen; and elimination of the infected subpopulation of T killer cells directed against the virus.

Considering that the TBE virus, as a rule, causes a noncytocidal infection of cells, it is logical to suppose that the infected immune system cells develop reversible functional defects, that are the basis of nonspecific modulation of the immunological reactivity in vivo.

Conclusions

1. In the case of acute infection, the TBE virus multiplies in various immune system organs: in the spleen, thymus, and bone marrow.

2. In the spleen the targets of the virus are cells with T-lymphocyte characteristics (that have the theta antigen and do not adhere to glass) and cells with macrophage characteristics (that do not have the theta antigen and do adhere to glass).

3. The virus multiplies only in those cells of the immune system in which the synthesis of DNA takes place; the activation of the cells by mitogens increases their sensitivity to the TBE virus.

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COMPARATIVE STUDY OF MAIN IMMUNOLOGICAL TEST USED FOR EVALUATION OF IMMUNITY TO TULAREMIA IN PERSONS HAVING HAD DISEASE VACCINATED AGAINST

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[Article by L. S. Kamennova, V. I. Pokrovskaya, , V. P. Vasil'yeva, L. I. Petrova, V. B. Lyubomudrov, Ye. V. Gavrilova, V. A. Golovanova, and A. Ye. Zhuravlev; Microbiology and Epidemiology Institute imeni N. F. Gamaleya of the USSR Academy of Medical Sciences, Moscow; and the Oblast Sanitary-Epidemiology Station, Novgorod]

[Text] For many years scientists have been studying the question of the duration of immunity in people who have been vaccinated against tularemia or who have had the disease; an effort is being made to find more sensitive methods for determining the duration of immunity. The literature contains contradictory data on the effectiveness of tests now being used to determine the length of immunity. The tularine skin test that is used extensively is the most convenient and the most easy to perform under any conditions because it is easier to set up and easier to evaluate its results in comparison to serological methods.

Many authors have indicated that when the vaccination is done properly, the allergic skin reaction in vaccinated individuals that appears after the tularine skin test remains for a fairly long period of time. It also appears earlier and disappears later than the antibodies that are found in serological reactions [4, 7-9, 11].

In the overwhelming majority of cases of individuals who have had tularemia, the body's sensitization to the tularemia antigen, which is revealed by the tularine skin test, remains positive for 10, 26 and 40 years after the illness and it often lasts for the individual's entire life [2, 9, 14].

In recent years, however, papers have appeared that show the allergic skin test lagging behind the serological reactions. Data have been published showing that 5 years after the vaccination the tularine test fails to reveal an average of up to 10-15 percent of the immuno-positive individuals [12]. Negative results have been obtained using the skin test on residents of northern regions and the Far East who have had positive serological reactions [1, 3, 15].

In a retrospective study of tularemia among populations in regions along the route of the Baykal-Amur Mainline in Khabarovsk Kray between 1977 and 1980, we did not obtain data confirming a weak allergic skin reaction in individuals who were natives of the Far East, such as the Nanays, Yakuts, and so on [5].

It is possible that the contradictions in the data obtained by different investigators who evaluated allergic and serological reactions to determine immunity among vaccinated individuals are tied to inaccurate documentation on the vaccinations themselves, the conditions under which the vaccine was stored, the inoculation techniques, the performance of the tularine tests, and the conditions under which the sera to be studied were stored and transported.

In order to study the question of correlation between the allergic test and serological reactions (the agglutination reaction and the passive hemagglutination reaction) in individuals vaccinated against tularemia or in individuals who have had the disease, between 1981 and 1982 we made a selective study of the population in 3 enzootic rayons in Novgorod Oblast (Novgorodskiy rayon, Chudovskiy rayon, and Malo-Visherskiy rayon). The existence of natural nidi for the infection in this territory has been described earlier; cultures of the tularemia microbe have been isolated from the water of local rivers a number of times over the course of many years and are still being isolated today; the last mass outbreak of the disease was reported in 1963-1964 [10]. Mass, systematic vaccinations with regular revaccination every 5 years have reduced the incidence of the disease to sporadic cases.

In studying the vaccinated individuals, we evaluated the results of immunity tests only in those people for whom there was precise documentation on the anamnestic response after inoculation. There were differences in the intervals and number of times that the local medical network performed the first and repeated (revaccination) vaccinations on the groups we studied. Taking into account the conclusions made by a number of investigators on the similarity among immunological indicators over recent years found in vaccinated and revaccinated individuals [6, 13], we calculated the time since immunization from the most recent date.

Materials and methods. Our study included the collection of anamnestic data, taking blood from the ulnar vein, and performing a tularine skin test, the result of which was evaluated after 48 hours; when there was a negative result, it was evaluated again after 72-96 hours. Blood serum to which sodium merthiolate had been added (1:10,000) as a preservative was placed in sterile ampules and kept in regular refrigerators at 4°C until being transported. The sera were sent to the Tularemia Laboratory at the Epidemiology and Microbiology Institute imeni N. F. Gamaleya over the course of 1 month following the time at which the blood sample was taken. Before being examined, the sera were kept frozen at -10°C. Over the course of 3 months after the blood was taken, all the sera were tested for the agglutination reaction and the passive hemagglutination reaction according to the generally accepted methods. Both reactions were carried out in dilutions of 1:10-1:1280. For the diagnosis we used a preparation with heightened sensitivity obtained from the "Schu" strain. We considered the latter dilution of the serum to have a positive result when we observed agglutination of ++++ or +++.

Results and discussion. We studied a total of 1028 individuals; 13 of whom had had tularemia before and 1015 of whom had been vaccinated in a various years. Of those vaccinated, 369 had been vaccinated once; 493 had been vaccinated twice; 127 had been vaccinated 3 times; 25 had been vaccinated 4 times; and 1 had been vaccinated 5 times. Out of all those who had been vaccinated, negative allergic and serological reactions were obtained in 28 people (2.7 percent); this included 27 people who had been vaccinated once and 1 person who had been vaccinated twice. Positive reactions (of any kind) were found in 987 of the vaccinated individuals (97.2 percent); 984 of those vaccinated (99.7 percent) had positive reactions in the tularine skin test; antibodies were found in the agglutination reaction test in 906 vaccinated individuals (91.8 percent); and antibodies were found in the passive hemagglutination reaction in 896 of those vaccinated (90.8 percent). A negative tularine test was found with a positive serological reaction in 3 of those who had been inoculated, that is, in 0.3 percent of those studied.

As the table indicates, the most effective test for determining immunity is the tularine skin test. Shortly after the inoculations, the allergic test remained positive in almost 100 percent of those who had been vaccinated; after longer periods of time, the number approached 100 percent. In the first years after inoculation both the agglutination reaction and passive hemagglutination reaction tests were positive in 90 percent of those studied; and at the maximum point, the agglutination reaction was positive in 94.7 percent and the passive hemagglutination reaction was positive in 92.1 percent. In subsequent years the serological indicators declined. In addition to vaccinated individuals, we also examined 13 people who had had tularemia not long before the study was done and also in the distant past. The number of cases were distributed as follows in terms of the amount of time that had passed since the illness: 4 months--1 person; 2 years--3 people; 18 years--1; 19 years--5; 33 years--1; and 36 years--2. All those who had had tularemia had a clear allergic reaction to the tularine skin test. The intensity of the skin reaction was clearly expressed in 5 individuals. In 2 of the people who had had the disease 19 years earlier we found hyperemia and infiltration areas reaching 4 x 5 cm in size, with mean antibody titers in the agglutination reaction and the passive hemagglutination reaction of 1:40 and 1:80. Strong positive allergic tests were also recorded for 2 people who had had the disease 36 years earlier: the size of the reactive skin areas (with infiltration and hyperemia) reached 4-5 cm with small necrotic changes in the center; antibodies were found in the agglutination reaction in just one of these individuals, with a titer of 1:20; antibodies were found in both individuals in the passive hemagglutination reaction in titers of 1:20 and 1:40. A strong positive allergic test was also observed in the individual who had had the disease 4 months earlier; this was combined with high antibody titers in both the agglutination reaction (1:640) and the passive hemagglutination reaction (1:1280). Besides these strong positive allergic tests, in one of the individuals who had had the disease 18 years earlier we observed a weak positive result in the tularine test (hyperemia and infiltration up to 0.5 cm) with a simultaneous absence of antibodies in the agglutination reaction and a very low titer of antibodies in the passive hemagglutination reaction (1:10). In the rest of the cases infiltration and hyperemia in skin sections in the tularine test did not exceed 1.5-2 cm. The average titers of antibodies in the agglutination reaction and the passive hemagglutination reaction were 1:10 and 1:80, only in those who had

had the disease 2 years earlier did we see antibodies in the agglutination reaction maintaining a titer of 1:160.

The effectiveness of immunological tests at various periods following vaccination

Срок после вакцинации (1)	Всего выявлено положительно реагирующих (2)	В том числе по данным (3)				
		тулярин. новой пробы (4)	РА (5)		РПГА (6)	
		%	%	титры (7)	%	титры (7)
5 дней—3½ мес (8)	114	100	94,7	1:10—1:320 / 1:30	92,1	1:10—1:640 / 1:39
1—5 лет (9)	773	99,7	92,0	1:10—1:160 / 1:24	91,0	1:10—1:640 / 1:26
6—10 лет (10)	73	98,6	90,0	1:10—1:80 / 1:21,1	85,0	1:10—1:320 / 1:24
11—25 лет (11)	27	100	85,0	1:10—1:80 / 1:22,6	92,6	1:10—1:80 / 1:24
(12) Итого . . .	987	99,7	91,8		90,8	

Note: The numerator represents the value of the positive results of antibody titration; the denominator represents the geometric mean of the titers.

Key:

1. Time since vaccination
2. Total with positive reactions
3. Data broken down by the various tests
4. Tularine test
5. Agglutination reaction
6. Passive hemagglutination reaction
7. Titers
8. 5 days-3.5 months
9. 1-5 years
10. 6-10 years
11. 11-25 years
12. Total

Conclusions

1. In a comparative study of the immunological reactions for determining the duration of immunity in individuals inoculated against tularemia, the largest number of positive reactions was detected by means of the tularine skin test (99.7 percent).

2. Antibodies were found in 91.8 percent of the individuals in the agglutination reaction and in 90.8 percent in the passive hemagglutination reaction.

3. A negative allergic test in the presence of antibodies in serological reactions was observed in 0.3 percent of the vaccinated individuals studied.

4. Among the individuals who had had tularemia in the past, an allegic skin test and antibodies in the passive hemagglutination reaction were recorded in

100 percent of the cases, regardless of the time since the disease (the maximum in this study was 36 years).

5. We observed a gradual decline in the titers of antibodies with the passage of time since the vaccination or the disease.

6. In order to determine the duration of immunity among an inoculated population when preventive measures are being implemented against tularemia, it is best to use the tularine allergic skin test.

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RESULTS OF EPIDEMIOLOGICAL SURVEY OF DIPHTHERIAL INFECTION IN 21 REGIONS OF RUSSIAN SOVIET FEDERATED SOCIALIST REPUBLIC

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[Text] Over the past 20 years large numbers of children have been given anti-diptheria inoculations and indicators of the incidence of this infection in the RSFSR reached their lowest level in 1975--0.03 cases per 100,000 people. However, starting in 1976 there has been an increase in the number of diphtheria cases in the republic; in 1981 indicators of the incidence of the disease in various regions ranged from 0.01 to 1.8 per 100,000 people, with an average indicator for the entire republic of 0.34. A rise in the incidence of the disease has been observed in all age groups, but the most intensive increase has been among adults. The mortality rate remains high.

In a previous paper [1] we discussed the need for an epidemiological survey of diphtheria. An epidemiological survey involves studying the incidence of diphtheria in a given region in terms of its dynamics, as well as monitoring the circulation of the agent of diphtheria among the population with the aim of determining the reasons for the spread of the disease and of making a prognosis. A survey also includes an examination of the immunological status of children, a study of the biological properties of the circulating strains, and finally, early detection of the disease.

Research methods and scope of research. An epidemiological survey of diptherial infection was carried out according to unified methodological recommendations between 1980 and 1981 in 21 regions of the RSFSR; immediately prior to the survey, a number of conferences and seminars were held in these regions for epidemiologists, pediatricians, infection specialists, and bacteriologists.

In order to study the immunological status of children in 9 regions we selected groups of children between the ages of 4 and 14 years, with 200 children in each group; they were given the Schick test in accordance with the set

instructions (a total of 39,186 tests were given). At the same time we checked to see that the inoculations were organized and performed properly.

The isolation of the diphtheria agent was done according to various indications; we examined groups of individuals who are at high risk of infection (residents of boarding schools, children's homes, dormitories at vocational-technical schools, etc.) The isolated strains were sent to the Diphtheria Laboratory at the Moscow Epidemiology and Microbiology Scientific Research Institute imeni G. N. Gabrichevskiy for a complete workup, including indentification of the variants' serotype, phagotype, and corycinotype. We made bacteriological studies of 741,278 people. A total of 1455 strains of corynebacteria taken from 1335 people from 927 nidi underwent identification.

With the aim of early detection of diphtheria, we did bacteriological studies of everyone suffering from angina; preparations were applied to their tonsils and the individuals were placed under active observation for the first 3 days following the treatment. These individuals were included in the group of people surveyed for diagnostic purposes.

Results and discussion. An analysis of our work showed that during the period of the study there was a change in the incidence of diphtheria in various administrative regions of the RSFSR. The number of cases in 6 administrative regions increased by a factor of 2-2.5; these regions were Saratov Oblast, Kuybyshev Oblast, Novosibirsk Oblast, Moscow Oblast, Maritime Kray, and the city of Moscow. In the other regions the incidence of the disease remained at its previous level, or there was an insignificant increase or decrease.

Compared to the period between 1970 and 1975, there was a drop in the proportion of children suffering from diphtheria and a rise in the proportion of adults with the disease. Children up to the age of 14 accounted for 24 percent of those with diphtheria in 1980; in 1981 they accounted for 18.3 percent. People 20 years and older accounted for 72.7 and 70.5 percent, respectively.

The determination of the children's immunological status showed that in the overwhelming majority of regions the number of positive reactions in the Schick test in various age groups ranged from 0.36 to 3.1 percent. Higher indicators were seen only in two oblasts: Kuybyshev Oblast with 8 percent among children 14 years of age; and Omsk Oblast with 6 percent among children 5 years of age. In an expanded study of children in these oblasts the percentage of positive reactions to the Schick test was lower than 5 percent. The number of questionable reactions to the Schick test in the oblasts ranged from 5.5 to 5.7 percent.

Consequently, the results of the Schick test in the oblasts studied indicate that children up to 14 years of age were in good condition in terms of their inoculation.

In a bacteriological study of the population by various groups, the isolation of diphtheria corynebacteria ranged from 0.71 to 1.6 percent (see table).

Carriers of diptheria corynebacteria in 16 administrative regions of the RSFSR*

Показания (1)	Число обследованных (2)	Выделено носителей коринебактерий, % (3)		Носительство коринебактерий по отдельным территориям, % (6)	
		всего (4)	из них токсигенных (5)	всего (4)	из них токсигенных (5)
С диагностической целью (7)	423 456	0,52	16,4	0,05 (г. Куйбышев) (8) 1,8 (Москва) (10)	1,3 (Новосибирск) (9) 27,1 (Саратовская область) (11)
По эпидемическим показаниям (12)	38 570	1,6	27,9	0,1 (Астраханская область) (13) 8,3 (Краснодарский край) (14)	12,5 (Краснодарский край) (14) 41,8 (Саратовская область) (11)
С профилактической целью (15)	279 252	0,71	2,2	0,007 (Пензенская область) (16) 1,3 (Саратовская область) (11) 6,3 (Омская область) (18)	4,2 (Саратовская область) (11) 6,0 (Приморский край) (17) 16,0 (Краснодарский край) (14)

*Five administrative regions did not submit data

Key:

1. Type of data
2. Number of individuals studied
3. Corynebacteria carriers isolated
4. Total
5. Toxigenic carriers
6. Percentage of corynebacteria carriers by region
7. For the purpose of diagnosis
8. The city of Kuybyshev
9. Novosibirsk
10. Moscow
11. Saratov Oblast
12. Epidemic indicators
13. Astrakhan Oblast
14. Krasnodar Kray
15. For the purpose of prevention
16. Penza Oblast
17. Maritime Kray
18. Omsk Oblast

As one would expect, the highest level of toxigenic corynebacteria carriers was found in studying epidemic indicators--27.9 percent. Among the large number of individuals examined for preventive purposes, only 2.2 percent of the corynebacteria carriers of diphtheria were toxigenic. The highest incidence of the disease was reported in Saratov Oblast, Novosibirsk Oblast, and Maritime Kray.

Among the strains that were obtained for identification, 30.2 percent were toxigenic corynebacteria strains. The virulent properties of the diphtheria agents, evaluated in terms of Dnm, ranged from 12.5 million to 100 million microbial cells. An average of 91.2 toxigenic strains had a Dlm between 25 and 50 microbial cells. Consequently, the pathogenic properties of the toxigenic corynebacteria remain at a high level and did not change from previous years. A total of 78.8 percent of the strains were of the gravis biotype, and 22.2 percent were of the mitis biotype. There was a sharp rise in the proportion of toxigenic strains of the mitis biotype in Moscow and in Maritime Kray (from 18 to 35.3 percent of the nidi).

In serological typing of the toxigenic strains included in the gravis biotype, 94.4 percent were divided into 2 groups; 26 percent of them were classified as belonging to serotype 2 and 68.4 percent to serogroup 27274. Of the nontoxigenic strains of the gravis biotype, 69.7 percent were also divided into 2 serogroups: 27274 and 23607.

Phagotyping of the toxigenic strains included in the gravis biotype resulted in subdividing these strains into 8 phagotypes. The most widespread phagotypes were OPQRSTg (between 30.4 and 59 percent of the nidi), ABg (between 17 and 53.3 percent) and ABfGH (between 7 and 23 percent). Nontoxigenic corynebacteria were subdivided into 2 phagotypes--ABCDfg and K; the first group included between 72.3 and 96.4 percent of these bacteria. No new phagotypes were found. The results of the phagotyping made it possible to make a more accurate determination of the sources of the infection and the pathways by which it spreads. We confirmed a correlation between the serological properties and phagolytic properties of diphtheria corynebacteria.

In 86.9 percent of the cases, there was agreement between the results of determining toxicity and the results of practical laboratory research; this correspondence was higher than it had been in previous years.

In the process of identification, in 13 percent of the cases a hyperdiagnosis was made in determining the toxicity of the strains; and in 0.1 percent of the cases, a hypodiagnosis was made. This demonstrates the possibility of overestimating the number of people with diphtheria and the carriers of toxigenic corynebacteria in the oblasts, and also the practical benefits of making a comparative identification of strains from various laboratories in the RSFSR.

In connection with organizing early detection of diphtheria in the population, there has been an increase in the proportion of people suffering from a localized form of diphtheria of the pharynx; it accounts for 80-85 percent of the cases. The incidence of the disease among inoculated children is characterized by a predominance of localized pharyngeal diphtheria (86 percent) and a tendency toward spontaneous recovery. Among nonimmune children, serious forms of the disease account for 56 percent of the cases and serious combined forms account for 34 percent of the cases. We found that hospitalization occurred at late stages: with 60 percent of those suffering from diphtheria seeking treatment during the first 2 days of the illness, only 23 percent of the children and 5-15 percent of the adults were hospitalized during this period. In 1980 a diagnosis of diphtheria based on data from a bacteriological examination of people suffering from angina was made in 66 percent of the cases; in 1981, in 64.5 percent of the cases.

In our study, in order to confirm a diagnosis of diphtheria in angina patients, along with a bacteriological culture of toxigenic corynebacteria we used Jensen's quantitative method, the results of which made it possible to narrow down the diagnosis: diphtheria or angina + carriers of toxigenic diphtherial corynebacteria.

Titration of the diptherial anatoxin using Jensen's method was performed in 1981 on 160 individuals with diphtheria and 35 angina patients. A comparison

of the clinical and serological data, taking into account the time over which the research was done, made it possible to diagnose angina with concurrent toxigenic corynebacteria carriers in only 5 patients with a preliminary diagnosis of "diphtheria?". This group included 3 children and 2 adults between 19 and 21 years of age. All the patients were inoculated against diphtheria.

It should be pointed out that the most difficult part of the epidemiological survey of diphtherial infection was early detection of individuals suffering from diphtheria. Active monitoring of angina patients over the first 3 days was not done in all the oblasts; it was limited to observing angina patients primarily in the city networks of children's polyclinics. Only in Maritime Kray was there active monitoring of children and adults in cities and rayons.

Thus, the epidemiological survey of diphtheria infection carried out for the first time in 21 administrative regions of the RSFSR helped increase the attention given by physicians to this infection, and it helped improve the quality of diagnoses and anti-epidemic measures. As a result of increasing the number of bacteriological examinations of angina patients, there was a more thorough diagnosis of those suffering from mild forms of diphtheria. This offers evidence in support of expanding epidemiological surveys of diphtheria infection throughout the entire territory of the republic, which is called for in Order No 575 issued on 10 September 1982 by the RSFSR Ministry of Health.

Conclusions

1. We found a high level of anti-diphtheria immunity among children up to 14 years of age: in various oblasts 96.9-99.4 percent of the children had a negative reaction in the Schick test.

2. The biological properties of diphtheria agents did not change during the observation period. Toxigenic diphtherial corynebacteria are characterized by a high level of pathogenicity.

3. The epidemiological survey helped in making a more thorough determination of diphtheria and of toxigenic diphtherial corynebacteria; and it helped to improve the quality of clinical and bacterialogical diagnoses.

4. Making a correct diagnosis of angina with concurrent toxigenic diptherial bacteria carriers is possible only by doing a serological examination of the patient using Jensen's method.

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METHODS FOR DIFFERENTIATING ROUGH (R) AND TRANSITIONAL (S-R) FORMS OF CHOLERA AND NONCHOLERA VIBRIONS

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 pp 98-99

[Article by I. I. Derteva, S. N. Tyumentsev, A. A. Popov, A. Kh. Kochetov, and O. S. Zabolotnaya; (Saratov, Irkutsk) in section "Problems in the Prevention of Zoonotic Infections in Regions of the North and Far East"]

[Text] The methods for differentiating rough (R) and transitional (S-R) forms of cholera and noncholera vibrions of Heyberg's group I are not precise enough. Diagnostic O-cholera sera, including adsorbed sera, often cause nonspecific agglutination of noncholera vibrions, which makes their identification even more difficult.

The goal of the present study was to determine specific indications that could be used to differentiate the rough and transitional forms of cholera and noncholera vibrions of Heyberg's group I that agglutinate in the presence of cholera O-serum.

For the experiment we took 4 strains (13-DV, 65-DV, 6-62-1DV, and 65) of noncholera vibrions in Heyberg's group I, used as adsorbents in the production of commercial diagnostic cholera sera. The noncholera vibrion strains were isolated from objects in the environment in the Far East; they were in the S-R phase of dissociation and agglutinated in the presence of O-cholera, plague, and normal equine sera.

Heating the vibrions at 100°C for 2.5 hours caused a sharp drop in their agglutinability or a complete loss of agglutinability.

The cholera vibrion strains used in the experiment were in the smooth (Vibrio cholerae 569B), transitional (V. eltor 7840), and rough (V. cholerae C-71) phases of dissociation. All the strains agglutinated in the presence of cholera sera up to a maximum titer of antibodies, but after boiling their agglutinability dropped to one-half to one-fourth the original level. Plague serum agglutinated the strains in titers of 1:100-1:200 (strain C-71 only after being heated at 100°C).

The cholera and noncholera vibrion strains were tested for their sensitivity to diagnostic cholera monophages in an agglutination reaction with antisera

obtained by immunizing rabbits with O-antigen according to the method described by Baker et al. (1946), in tests of diffusion precipitation in gel, immunoelectrophoresis, and immunoluminescence.

The most clearly defined differences between cholera and noncholera vibrions were seen in the diffusion precipitation reaction in gel. Cholera vibrions in various forms of dissociation formed 2 zones of precipitation with homologous cholera O- and PO-sera: there was a sharp line along the alveolus with antigens, and a less distinct line turned toward the alveolus with the serum. The first precipitation line is apparently formed by the 1-O somatic antigen; the seond is formed by nonspecific precipitinogens. Noncholera vibrions form only one, nonspecific zone of precipitation. The antiserum to the 6-62-1-DB noncholera vibrion strain formed a line of precipitation not only with a homologous culture, but also with the noncholera vibrion strain 65.

Differences between the cholera and noncholera vibrions have also been observed in immunoelectrophoretic analysis. As a result of the interaction among the S- and R-forms of the cholera vibrions and the the O-cholera and PO-sera respectively, in the cathode section of an agar layer single bands were formed which were absent in tests on noncholera vibrions.

In the immunoluminescence analysis, specific luminescence of microbial cells and bacilli was seen in smears taken from cultures of cholera vibrions in the S- and S-R phases of dissociation. In smears taken from cultures of the cholera vibrion in the R-form, single microbial cells were luminescent. None of the cells from any of the strains of noncholera vibrions tested showed any luminescence.

An analysis of the results of cross agglutination reactions by antisera, obtained by immunizing rabbits with O-antigens of cholera dissociants and noncholera vibrions, allowed us to conclude that there is no antigenic relation between them.

Cholera vibrions in the smooth and transitional phases of dissociation were lysed by homologous cholera monophages up to DRT [expansion unknown]; and the R-variant of the cholera vibrion (C-71) in dilutions lower than DRT. Noncholera vibrions showed resistance to cholera monophages.

Thus, for identifying cholera vibrions in the transitional (S-R) and rough (R) phases of dissociation, we can recommend additional tests, primarily the reaction of diffusion precipitation in gel and immunoluminescence.

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INDICATORS OF NONSPECIFIC REACTION IN EXPERIMENTAL ANIMALS TO INTRODUCTION OF VARIOUS DOSES OF CHOLERA VACCINES

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[Article by M. S. Naumshina, T. V. Bugorkova, and A. K. Adamov (Saratov)]

[Text] Over recent years a fairly large body of experimental material has been accumulated on the integration in immunogenesis of antibodies and various metabolic cycles.

Changes in nonspecific, biologically active substances in the serum and cells of the macrophage-mononuclear system of the macroorganism, as a rule, precede the appearance of specific immunoglobulins; the strength of the reaction corresponds to the reaction-producing capacity of the vaccine preparations. According to data found in the literature, it is well known that the corpuscular vaccines with a greater reaction-producing capacity compared to the cholerogen anatoxin, cause a more pronounced accumulation of corticosteroid hormones in the blood (Gor'kova et al., 1972; Tropina et al., 1978; etc.); they cause a change in energy metabolism (Lyusid et al., 1972; Ledvanov et al., 1977; etc.) and in several lysosomal enzymes (Krupenina, 1969; Loktev et al., 1974; Slepukhin et al., 1976).

The aim of our study was to find the earliest and most informative test for evaluating the reaction-producing capacity of cholera vaccines.

We used 2 vaccines in our experiments: the cholera corpuscular vaccine and a chemical vaccine (cholerogen anatoxin); and we used 2 types of animals: chinchilla rabbits and mixed-breed guinea pigs. The vaccine was introduced subcutaneously one time in quantities equal to 1, 10, 100, and 1000 man-doses per 1 kg of each animal's body weight. On the first and third day following the vaccination we checked the serum and tissues for activity of the complement system, lysozyme, lipase, fructose-1,6-diphosphate aldolase, acid and alkaline phosphatase, catalase, and other enzymes.

The experimental data showed that several biologically active systems and the activity of various enzymes depended to a certain extent on the type of experimental animal, the dose and antigenic composition of the vaccine, and on the amount of time that had elapsed since the preparation had been introduced.

In guinea pigs, the most informative source of data was the changes in the activity of complement, lysozyme, and lipase on the first day following immunization that were evident when compared to the data obtained from the same animals prior to vaccination. For example, when 1 man-dose of corpuscular cholera vaccine was introduced, we observed a significant increase in the activity of the enzymes in almost all the animals: the lipase activity increased in the serum of all the animals; the complement activity increased in 88 percent of the animals; lysozyme activity increased in 56 percent of the animals, while in 44 percent of the animals the activity did not change. The arithmetic mean indicators of the increase in enzymatic activity from 10 experiments in each group were statistically significant.

With a 10-fold increase in the dose of the vaccine, we recorded a drop in the lipase activity in all the animals; there was a drop in the complement activity in 22.3 percent of the animals, while in 77.7 percent of the animals there was no change. Lysozyme activity decreased in 50 percent of the animals, while in the rest the activity stayed within the original values. With a 100-fold increase and even a 1000-fold increase, we observed approximately the same suppression of enzymatic activity as that caused by introducing 10 man-doses.

When we immunized the guinea pigs with cholerogen anatoxin, there was a suppression of enzymatic activity. For example, 1 man-dose of the vaccine caused a suppression of lipase activity in all the animals, a suppression of complement activity in 50 percent of the animals, and a suppression of lysozyme activity in 60 percent of the animals. Complement activity increased in 4 out of 8 guinea pigs and lysozyme activity increased in 1 out of 10 guinea pigs. With a 10-fold increase in the vaccine dose, on the first day complement activity declined in all the animals; lysozyme activity decreased in 80 percent of the guinea pigs; lipase activity rose above the original level in 3 animals, while it was lower in 5 animals, and in 2 animals it did not change at all.

Immunization of guinea pigs with 100 man-doses caused an increase in lipase activity in all the animals; an increase in complement activity in 50 percent of the animals; and an increase in lysozyme activity in 40 percent of the animals. In the experiments with 1000 man-doses of cholerogen anatoxin we obtained unstable results.

We studied the activity of enzymes (lipase, lysozyme, complement, aldolase, amylase, acid and alkaline phosphatase), as well as the serum protein and sugar content in rabbits before immunization and 1, 3, and 10 days following a single introduction of 1, 4, 16, 50, and 100 man-doses of corpuscular cholera vaccine. The results that we obtained were not statistically significant for any of the enzymes studied.

Thus, the optimal immunization dose of corpuscular cholera vaccine, which is equal to 1 man-dose per 1 kg of the animal's body weight, brings about a significant increase in the activity of lipase and complement; when the dose is

increased 10-fold or more, there is a suppression of enzymatic activity. Chemical cholera vaccine (cholerogen anatoxin), when introduced in a quantity equal to 1 man-dose, causes suppression of enzymatic activity; when introduced in a quantity equal to 100 man-doses, it causes stimulation of lipase, complement, and lysozyme activity.

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STABILITY OF HEMOLYTIC TEST IN STRAINS OF VIBRIO CHOLERAE ELTOR OF VARYING VIRULENCE

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[Article by O. V. Osaulenko, A. S. Maramovich, and A. A. Veyde (Irkutsk)]

[Text] The complex method for determining the virulence of V. cholerae eltor includes a study of hemolytic activity. Data in the literature indicate that this characteristic is not always constant (Nikitina and Uryupina, 1974; Sakazaki et al., 1978). Stogova and Semiotrochev (1979) confirmed that hemolytic data cannot be used to determine the virulence of cholera vibrions because of the lack of uniformity in terms of hemolytic activity among the various strains.

We studied the uniformity of a population of V. cholerae eltor of varing origin with respect to hemolytic activity and the correlation between the results and the composition of the population.

In the experiments we used 16 virulent, 16 mildly virulent, and 31 avirulent strains of V. cholerae eltor from the museum collection of live cultures from the Irkutsk Antiplague Institute.

The virulent vibrions were typical in terms of their cultural, morphological, and serological properties; they were sensitive to the eltor phage and the virulence phages; they were cholerogenic in baby rabbits and they were nonhemolytic.

The mildly virulent and avirulent strains that originated in water agglutinated in diagnostic titers of the specific serum and the corresponding typical serum; they were lysed by the eltor phage and in nondiagnostic titers, by the CDP_3 [expansion unknown], CDP_4, and CDP_5 phages; on sheets of alkaline agar they formed colonies with a whitish coloring; when they were recultured they formed semi-transparent and opaque clones. Subcultures from these colonies did not differ in terms of their basic biological properties. The mildly virulent strains caused death in isolated animals, sometime with signs of diarrhea; in the large intestine of the baby rabbits we observed an accumulation of an opaque, stained fluid. The avirulent strains did not cause any illness among the baby rabbits or any pathological anatomical changes.

The composition of the population in terms of hemolytic activity was studied in a solid medium according to a method that we modified; it was studied in a fluid medium according to Greig's method. We performed Greig's test in a meat and peptone broth. In the positive cases we observed complete dissolution of erythrocytes and partial hemolysis, when part of them remained unlysed and settled on the bottom of the test tube like a "cloud." In a negative reaction all the erythrocytes fell to the bottom of the test tube. Using Greig's method we studied no more than 100 colonies of each strain at one time. When we studied the hemolytic activity in a solid medium, the hemolytic clones developed on the surface of the agar and in the middle of its colony with a pink translucent zone. If the population of hemolysis-positive strains was not homogeneous in terms of the aspect under study, then on the agar and deep in the middle of the hemolytic colonies we found colonies lacking this property.

The results of our research showed that the population of virulent strains of V. cholerae eltor is homogeneous in terms of hemolytic activity in a 3 percent blood agar and in a fluid medium.

The population of mildly virulent strains of V. cholerae eltor turned out to be nonhomogeneous in terms of hemolytic activity. Of the 690 colonies studied using Greig's test, 360 exhibited partial hemolysis and 333 complete hemolysis. Efforts to isolate the nonhemolytic colonies using Greig's test were unsuccessful. However, in a study of $1.2 \cdot 10^4$ colonies in a 3 percent blood agar we found 5 nonhemolytic colonies from the same strain. When we subjected them to further study using Greig's test to determine their hemolytic activity, their ability to lyse sheep erythrocytes was restored.

The mildly virulent strain V. cholerae eltor I-703 is of the greatest interest. When we studied $1.2 \cdot 10^4$ colonies of the original strain in blood agar, we isolated 10 nonhemolytic colonies; in a Greig's test applied at the moment of isolation, they were hemolysis-negative, but after double passage in nutrient media their hemolytic activity was restored. The results of studying the composition of the population in terms of hemolytic activity of subcultures of the I-703 strain of V. cholerae eltor, which had passed repeatedly through the intestines of baby rabbits, showed that the population does not contain hemolysis-negative cells that maintain this property on a stable basis. As a result of research on $0.6 \cdot 10^4$ colonies of the subculture from the 14th passage, we found 42 hemolysis-negative colonies. In a second determination of hemolytic activity using Greig's test and in a solid medium, we found only 4 hemolysis-negative colonies that maintained this property on a stable basis, and demonstrated the capability of being lysed by virulence phages in DRT and of having a cholerogenic effect in baby rabbits.

When we used Greig's test to study an entire population of selected, newly isolated anti-virulent strains of the vibrion, we found that 23 strains were hemolysis-positive and 3 were hemolysis-negative. Nonhemolytic strains grew in the form of opaque colonies, which after a two-fold recultivation, split into opaque and semi-transparent colonies. Both types of colonies lacked hemolytic activity. In a second study of 53 colonies, 17 were nonhemolytic. However, in subsequent recultivation of the hemolysis-negative cultures the hemolytic properties appeared and remained stable in Greig's test and in a blood agar. The population of avirulent strains was not homogeneous in terms

of their ability to lyse sheep erythrocytes. Of $5.2 \cdot 10^4$ colonies, 59 did not form a hemolysis zone in a blood agar. In a study of the hemolytic activity of these colonies using Greig's test, we obtained positive results.

Finkelstein (1966) believes that the transparent hemolytic variety arises in the primary nonhemolytic population that forms opaque colonies. Our observations correspond to these data. When isolating nonhemolytic vibrions from the environment, their population can split into hemolysis-negative semi-transparent and opaque colonies, which when passed through nutrient media take on hemolytic properties.

Therefore, the simultaneous study of no fewer than 10^3 colonies of strains of V. cholerae eltor in a blood agar makes it possible to determine the population composition of the given culture and isolate the hemolysis-negative colonies. Fluctuations in hemolytic activity in subcultures of the same strain indicate that the population is not homogeneous and they are characteristic of mildly virulent and avirulent strains of V. cholerae eltor. When using a complex method to make an objective evaluation of the virulence of strains of V. cholerae eltor taken from the environment and lacking hemolytic activity at the time of isolation, one must study the stability of the hemolytic test.

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PHAGOTYPING OF ENTEROPATHOGENIC NONAGGLUTINATING VIBRIONS ISOLATED IN SIBERIA AND FAR EAST

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[Article by V. S. Ganin, N. F. Bystryy, L. Ya. Urbanovich, A. F. Pinigin, E. G. Nabiyev, A. S. Maramovich, Yu. S. Musatov, Yu. M. Sheremet (Irkutsk, Saratov, Chita, Khabarovsk)]

[Text] There is a large group of microorganisms that exist in nature and belong to the vibrion family, but do not agglutinate in the presence of O-cholera serum (NAG-vibrions). It has been determined (Dunayev et al., 1971, 1976; Goncharov et al., 1975) that certain serotypes of NAG-vibrions can cause acute gastro-intestinal illnesses in humans.

The differentiation of NAG-vibrions that are enteropathogenic to humans (especially those isolated from the environment) from nonpathogenic vibrions has presented certain bacteriological problems. With this differentiation as a goal, a method of serological typing was developed for enteropathogenic NAG-vibrions based on the nonhomogeneity of their O-antigen (Sakazaki et al., 1970; Adamov et al., 1974; Yermol'yeva et al., 1974; Krasnova et al., 1974; Somova et al., 1976; Ganin et al., 1978; Khaytovich et al., 1980). A serological examination of NAG-vibrions showed that it is not always possible to determine the serotype in patients with or carriers of NAG-infection, since there are obviously enteropathogenic NAG-vibrions of as yet unknown serotypes. In light of this, phagotyping of enteropathogenic NAG-vibrions can add substantially to the results of serological typing.

Phagotyping of NAG-vibrions was done using experimental phages TEPV-1, 2, 3, 4, 5, prepared at the "Microbe" All-Union Antiplague Institute in Saratov by means of the agar layer method. Ten-fold dilutions of phages (from an undiluted titer to a diagnostic working titer) were prepared in Marten's broth (pH 7.2-7.6) and placed in a culture ground using an automatic applicator, after which the dishes were incubated at 37°C for 16-18 hours.

The lytic action of the phages was evaluated according to a 4 point system. The presence of a lytic stain at the point of application of a phage from any dilution with a degree of lysis not less than 2 pluses was considered a positive result.

Strains of NAG-vibrions were isolated at different times in Kurgan, Kemerovo, Novosibirsk, Omsk, Irkutsk and Chita oblasts, in Altay, Krasnoyarsk, and Maritime krays, and in the Yakutsk ASSR.

We established a phagotype for 651 (or 31.4 percent) of the 2075 strains of NAG-vibrions isolated from humans and from objects in the environment. We found phagotypes more often for those strains isolated from humans than for those isolated from objects in the environment (43.5 and 29.6 percent, respectively). Of the 262 strains isolated from patients and carriers, 108 belonged to phagotype groups I, II, III, or IV and only 6 strains belonged to mixed groups (I and II or I and IV). We did not find any strains belonging to phagotype V among those NAG-vibrions isolated from humans. Phagotype I was the dominant group among these NAG-vibrions, and phagotypes IV and III were the second and third most common.

We established a phagotype for 537 (29.6 percent) of the 1813 strains of NAG-vibrions isolated from objects in the environment. These NAG-vibrions belonged to 5 different phagotypes (I, II, III, IV, and V) and to 7 mixed groups (I and II; I, II and IV; I and IV; I and V; II and IV; II and V; IV and V). Phagotype I was the predominant group among these NAG-vibrions, just as among the strains isolated from humans. Phagotypes I [as published] and IV were the next most common types found among these vibrions.

An analysis of the results of phagotyping showed that in Siberia and the Far East, enteropathogenic NAG-vibrions are isolated from patients with diarrheal illnesses and carriers of such illnesses, and these NAG-vibrions are also widespread in objects in the environment.

The isolation of NAG-vibrions of identical phagotypes from patients with clinical signs of gastro-intestinal illness, from healthy carriers, and from objects in the environment (open reservoirs), indicates that it is possible to be contaminated with an NAG-infection by using water. As a rule, the anamnestic response in patients with acute gastro-intestinal illnesses caused by NAG-vibrions has been the result of using unboiled water for drinking and household purposes.

NAG-vibrions apparently belong to a certain phagotype on a stable basis, since when the same patients with an NAG-infection were re-examined, we isolated NAG-vibrions of the orginal phagotype.

The method of phagotyping NAG-vibrions using TEPV phages makes it possibe to determine the ecological and geographical limits of their distribution, to identify the phagotypes of NAG-vibrions that cause acute gastro-intestinal illnesses among humans, to reveal epidemiological connections with the source of infection, and to determine the paths by which it spreads.

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ULTRASTRUCTURE OF R- AND S-VARIANTS OF BRUCELLA OVIS

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 p 102

[Article by T. A. Tarasova, L. P. Repina, A. I. Shcherbakov, Ye. P. Golubinskiy, and A. F. Pinigin (Irkutsk)]

[Text] The use of electron microscopy technology has made it possible to begin a more in-depth study of the morphology of bacteria. The literature contains information on the ultrastructure of R- and S-variants of B. melitenis, B. abortus, and B. suis. We have not encountered any data on the submicroscopic structure of B. ovis in the available literature. We have studied the ultrastructure of B. ovis and its subcultures in the smooth form. We used the I-65 strain of B. ovis for our research, which was isolated at a nidus of infectious epidydimitis among sheep in the Tuva ASSR. At the same time we studied S- and R-variants of reference strains of B. melitensis 16 M, B. abortus 544, and B. suis 1330.

The smooth variant of B. ovis was obtained by growing the original strain in nutrient media with aniline stains. The rough variants of the reference strains of brucella were isolated by selecting colonies that had been stained according to the White-Wilson method.

The original brucella cultures and their subcultures were studied by means of tests designed to differentiate the different types of brucellae. Strains in the rough form agglutinate well in a trypaflavine solution and they have a positive reaction to thermoprecipitation. All the colonies that we grew were stained a dark blue color in a vital stain using crystal violet. In terms of its biological properties the strain being studied is identical to reference strains of B. ovis from Australia, New Zealand, and Argentina. Unlike other brucellae in the rough form, the B. ovis I-65 grows only in nutrient media to which native bovine or equine serum has been added and that have an increased carbon dioxide content.

The original strains of B. melitensis, B. abortus, and B. suis had characteristics typical of those brucellae and in terms of its biological properties, the S-subculture of B. ovis resembled B. melitensis. For the electron microscopy study we used a 2-day culture of brucellae that had been grown in Marten's agar with a pH of 6.8. We used the method described by Ryter et al. (1958) to fix and treat the material. The sediment of brucellae that we

obtained was dehydrated in alcohol and then covered with a mixture of epoxy resins (epone 812 and araldite M). Ultrathin sections, prepared on an LKB ultratome, were contrasted with uranylacetate and lead citrate and examined with an IEM-100S electron microscope.

We did not find any differences in the submicroscopic structure of the original (rough) B. ovis cells on the one hand and of the smooth B. melitensis, B. abortus, and B. suis cells on the other hand. The B. ovis cells, like other types of brucellae, have a cell wall with a convoluted outer membrane that is 8-10 nm thick and consists of 3 layers that are each 2.5-3.5 nm thick. The cytoplasmic membrane is 7-8 nm thick and also has 3 layers, each of which is approximately 2.5 nm thick. Between the outer membrane of the cell wall and the cytoplasmic membrane there is an osmiophobic layer whose thickness ranges between 3 and 15 nm. The mesosomes often have the form of elongated round loops that are formed by the cytoplasmic membrane. The cytoplasm is granular due to the ribosomes that are distributed uniformly throughout, but are sometimes grouped closer to the center of the cytoplasm. The nucleoid is irregular in form and in the majority of cells it is located in the central part of the cell; in some cases it appears as a diffuse formation.

The population of B. ovis cells in smooth form was heterogeneous in terms of its composition. The majority of cells (75 percent) were similar to the cells in the original rough culture. The rest of the cells (25 percent) differed from the genetically similar rough variants and the smooth forms of other types of brucellae in that they had mesosomes that were more varied in form and a "capsule-like substance" surrounding the outer membrane of the cell wall. This substance takes the form of a uniform layer up to 15-20 nm thick or of protuberances up to 45-50 nm high. A similar cellular structure is also seen in the rough variants of sheep, cow, and pig types of brucellae.

An electron microscopic study of brucella cells showed that the ultrastructure of original rough strains of B. ovis and smooth B. melitensis, B. abortus, and B. suis did not differ substantially. These cells have a clearly defined cell wall with a smooth surface on the outer membrane. S-subcultures of B. ovis and R-variants of other types of brucellae obtained under laboratory conditions were found to have cells containing a capsule-like substance.

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BIOLOGICAL DESCRIPTION OF BACILLI ISOLATED IN SOIL NIDI OF ANTHRAX IN CHITA OBLAST

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[Article by L. N. Yuzvik, P. I Naymanov, and Yu. S. Musatov (Chita, Irkutsk)]

[Text] With the aim of evaluating the epizootological and epidemiological characteristics of Chita Oblast with regard to anthrax, we conducted a bacteriological study of 358 soil samples taken from various sites consistently unfavorable in terms of this infection. Samples were taken at 119 sites from the surface and at a depth of 20-40 cm. The most recent cases of anthrax in humans and livestock in these areas were reported in 1958.

The basic test used to isolate the strains was the anthrax phage test, which unlike the biological method, makes it possible to isolate mildly virulent and avirulent strains.

According to the results of this test, we isolated 15 strains lysed by anthrax phage K (from the All-Union Experimental Veterinary Science Institute) and Gamma (from the Moscow Veterinary Academy). The degree of lysis varied from clearly pronounced to just isolated small points.

The morphology of growth in liquid and dense media corresponded to the R-form. In the microbiological tests, we used the B. anthracis (STI) [expansion unknown] and B. cereus T. strains as the control groups.

The vegetative cells of 12 strains stained with luminescing anthrax serum gave off a specific luminescence, which we evaluated as ++++. The luminescence of the other strains was less intense (++). We set up a precipitation reaction (Askoli) using cultures of the strains being studied that had been killed by heating and then filtered. Positive results were recorded for 12 strains. The precipitation reaction using a culture of the standard strain B. cereus T was negative.

The result of the test using penicillin (the "pearl necklace" test) was evaluated as mildly positive in 13 strains. All the strains had a positive reaction to lecithinase and phosphatase. Individual cells of the strains we studied form a poorly defined capsule. The strains form spores well. The spores are centrally located in the cell. The cultures of the strains we

studied are not pathogenic for white mice. The original culture is discharged from the organs of the test animals between the fifth and seventh day.

When white mice are infected with a culture of vegetative cells (500 million in 0.5 ml) with chicken yolk, death follows after 20-24 hours with thorough involvement of all the organs.

Thus, the results obtained in a study of isolated strains do not allow us to identify precisely their place in the taxonomy of the bacilli. We are inclined to classify some of them as mutant, avirulent forms of the anthrax agent. The literature does contain data on isolating similar nonpathogenic strains of the anthrax agent from the soil and other objects in the environment (Yaremenko et al., 1976; Lebedinskaya et al., 1978). The epizootological role of these strains is unclear. Therefore, research needs to be done to study the possibility of reversion in the strains with a restoration of the determinant of virulence.

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SENSITIVITY OF BRUCELLAE ISOLATED IN TUVA ASSR TO ANTIBIOTICS

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[Article by T. G. Charnaya, A. F. Pinigin, and L. P. Repina (Kyzyl, Irkutsk)]

[Text] The study of the sensitivity of agents of various diseases to antimicrobial preparations is the basis for choosing the most rational system of treatment.

We determined the sensitivity of 72 strains of Brucella melitensis, 9 strains of B. abortus, and 7 strains of B. ovis, all isolated in the Tuva ASSR, to 16 antibiotics. We used the method of serial dilutions in dense nutrient media. We used benzyl penicillin, oxacillin, methicillin, ampicillin, bicillin, streptomycin, kanamycin, levomycetin, tetracycline, monomycin, gentamicin, polymyxin M, erythromycin, lincomycin, ristomycin, and morphocycline.

We found that all the strains of B. melitensis and B. abortus have a high degree of sensitivity to gentamicin, streptomycin, and kanamycin (the minimum inhibiting concentration for the majority of the strains was less than 1.0 μg/ml; the strains were less sensitive to tetracycline (the minimum inhibiting concentration was 1.25-5.0 μg/ml). Benzyl penicillin, methicillin, bicillin, levomycetin, and erythromycin had a weak bacteriostatic effect on the brucelli isolated in Tuva (the minimum inhibiting concentration was 15-100 μg/ml). All the strains of B. melitensis and B. abortus were resistant to oxacillin, polymyxin M, and ristomycin (the minimum inhibiting concentration was over 200 μg/ml).

Strains of B. ovis were highly sensitive to tetracycline, streptomycin, and monomycin (the minimum inhibiting concentration was 0.4-0.1 μg/ml). Benzyl penicillin, kanamycin, morphocycline, ampicillin, and lincomycin had a less pronounced bacteriostatic effect (the minimum inhibiting concentration was 5.0-10.0 μg/ml).

An analysis of the data we obtained allows us to conclude that the brucellae isolated in smooth and rough forms in the Tuva ASSR differ in terms of their resistance to antibiotics. B. ovis was the most sensitive to antibiotics.

The sensitivity of B. melitensis and B. abortus isolated in Tuva to the majority of the antibiotics tested does not correspond to the sensitivity of

typical strains of brucellae;this indicates that the strains we studied are unusual. For example, the minimum inhibiting concentration of benzyl penicillin, levomycetin, and erythromycin for these strains of B. melitensis and B. abortus were 10-30 times higher than the values described by Navashin and Fomin (1974). The strains isolated in Tuva were resistant to oxacillin, polymyxin M, and ristomycin.

The data we obtained may be of practical significance. It is well known that in treating people suffering from brucellosis, in addition to using antibiotics of the tetracycline series, levomycetin and erythromycin are also recommended. It is clear than under conditions in the Tuva ASSR, the use of these antibiotics is not desirable, since they have a weak bacteriostatic effect. At the same time, gentamicin, kanamycin, and streptomycin are very effective against the brucellae that have been isolated in the Tuva ASSR and should play a leading role in the treatment of brucellosis patients in this region.

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DESCRIPTION OF STRAINS OF YERSINIA ENTEROCOLITICA ISOLATED FROM HUMANS AND ANIMALS IN SIBERIA AND FAR EAST

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[Article by L. A. Timofeyeva, L. P. Mironova, V. Ya. Golovacheva, N. S. Gaydukova, O. A. Mikhaylova, N. M. Busoyedova, and K. V. Podbolotov (Irkutsk, Khabarovsk, Vladivostok, and Yuzhno-Sakhalinsk)]

[Text] The present paper summarizes data on strains of Y. enterocolitica isolated by associates of antiplague institutions and departments of especially dangerous infections at sanitary-epidemiological stations in Siberia and the Far East between 1971 and 1980 and studied at the Microbiology Department of the Irkutsk Antiplague Institute.

The first cultures were isolated in 1971 on Sakhalin Island from a gray rat and a red-and-gray vole. Over recent years 264 strains have been obtained in Sakhalin Oblast. Similar cultures have been reported in Maritime (125), Khabarovsk (73), and Krasnoyarsk (23) krays; in Kamchatka (63), Irkutsk (9), Chita (2), and Novokuznetsk (1) oblasts; and in the Buryat ASSR (16) and Yakutsk ASSR (13). A total of 589 strains have been studied over 10 years.

The overwhelming majority of strains (354) were isolated from rodents; 78 were isolated from humans; 44 from other types of animals; 20 from arthropods; and 17 from birds. A large number of strains (120) were isolated from rinses of food products and from objects in the environment. The cultures from humans were obtained mainly from people who had diagnoses of hepatitis, German measles, pseudotuberculosis, enterocolitis, appendicitis, etc.

The isolation of cultures was recorded over the course of the whole year, but primarily during the autumn, winter, and spring seasons.

The overwhelming majority of strains were isolated by keeping the material in a storage medium (phosphate buffer, Serov's medium) in a refrigerator at 5°C with subsequent cultivation in agar.

In smears of the agar and broth cultures, Y. enterocolitica microbes appear as short bacilli with rounded ends. The bacteria are gram-negative; they are motile at 20-22°C and nonmotile at 37°C. Colonies that are round in shape with a smooth surface and even edge grow in agar media after 24-48 hours. In

various strains the colonies have a granular or slightly bumpy surface and an uneven, indented edge. Cultures in broth have a regular opacity and on the second or third day they form a ring alongside the wall on the surface of the medium. At a temperature of 28°C the cultures are not lysed by plague and pseudotuberculosis bacteriophages; at 37°C, 8 of the strains studied were sensitive to the pseudotuberculosis bacteriophage when tested in a solid agar medium.

On the first or second day the strains break down glucose, galactose, maltose, trehalose, levulose, mannose, mannitol, and with few exceptions, cellobiose, glycerine, sorbitol, and inositol. The majority of cultures studied are capable of breaking down arabinose, xylose, sucrose, salicin, and esculin. Some of the strains break down rhamnose and raffinose. A few strains form an acid in the presence of melibiose. The breakdown of lactose did not occur during the first 3 days of observation; during later periods (between 4 and 17 days) the formation of an acid was observed in some strains. The strains do not break down adonite, dulcite, or inulin. When grown in Hiss' medium, the majority of cultures form a gas of small bubbles in the presence of 1-2 or more substrates. The strains break down urea and change the color differential medium (Timofeyev et al, 1957) to blue and the universal graduated column (Serov, 1978) to yellow with a brownish stripe along the injection line.

The majority of strains grown in Hottinger's broth (pH 7.2, the depth of the split in the protein molecule is 60-70 percent, and the aminic nitrogen content is 150 mg percent) form hydrogen sulfide. Indole formation and the ability to reduce nitrates into nitrites was observed in more than 90 percent of the strains. All the strains had a positive reaction to catalase, ß-galactosidase, methyl red, and Foges-Proskauer at 22°C and a negative reaction to oxidase and phenylalanine desaminase. The strains decarboxylate ornithine and they do not possess lysine decarboxylase or arginine dihydrolase. In determining the hemolytic activity, positive results were obtained in Hottinger's broth. Of the 431 strains studied, 68 percent demonstrated lecithinase activity.

The strains are resistant to penicillin and they show penicillinase activity. In a study to determine the interaction between Y. enterocolitica and other antibiotics (using the method of serial dilutions in agar), it was established that the strains are most sensitive to tetracycline, oxytetracycline, and morphocycline; they are less sensitive to rondomycin, sigmamycin, kanamycin, streptomycin, and neomycin. The strains are not sensitive to benzyl penicillin, ampicillin, and ristomycin. Growth in the culture was observed when the concentration of the latter was 187 µg/ml.

The strains are not pathogenic in guinea pigs when introduced subcutaneously in a suspension of the culture containing a dose of $1 \cdot 10^9$-$3 \cdot 10^9$ microbial cells. After 820 white mice were infected subcutaneously with a 0.5 ml 24-hour broth culture (360 strains), 84 animals died. Most of the white mice died on the first or second day. Of 235 white mice infected intra-abdominally, 62 died. When the material was applied to the animals' skin, none of them died. When a culture containing a dose of 10^9 microbial cells with a suspension of dried chicken yolk was introduced into white mice, the intestinal yersiniosis did not cause death in any of the animals, but the preparation does cause a sharp

increase in the sensitivity of white mice to plague and pseudotuberculosis microbes, and listeriosis and erysipelas agents.

The strains we studied correspond in terms of basic biochemical properties to strains isolated from humans and animals that were graciously provided to us by Professor Molliar from France, Professor Knapp from the FRG, and Doctor Rakovskiy from Czechoslovakia. This allows us to assume that rodents are one of the sources through which humans are contaminated by intestinal yersiniosis; convincing proof of the role of rodents in pseudotuberculosis infection has been provided by many investigators.

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COMPARATIVE EXPERIMENTAL AND MORPHOLOGICAL DESCRIPTION OF PATHOGENICITY OF VIBRIO ELTOR, ISOLATED FROM OPEN RESERVOIRS

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[Article by S. G. Sappo, V. S. Kolesnik, and A. S. Maramovich (Irkutsk)]

[Text] We used 3 strains of V. eltor isolated from open reservoirs that are epidemically favorable in terms of cholera. Two of these strains (I-935 and I-723) are mildly virulent and the other (I-949) is avirulent*. A culture of each strain was introduced into animals intra-intestinally in doses of 10^5 and 10^7 vibrions per 100 kg of the animal's body weight. For each of these doses we used 10 baby rabbits, which were then studied pathomorphologically and bacteriologically after 12, 24, 48, and 72 hours. We also examined the animals that died and those that were put to sleep during the agonal period of the disease.

In the dissection of the animals the pathological anatomical changes were characterized by hyperemia of the intestinal serous membrane with the intestine containing a moderate amount of liquid or viscous material; the large intestine was enlarged and filled with a semi-transparent, opaque yellowish, or yellow fluid. In addition, there was also moderately pronounced dystrophy of the liver and kidneys. All these changes were more marked in the baby rabbits that had received the culture containing the I-935 strain, and they were found consistently among those that were given the 10^7 dose and less often among those that received the dose of 10^5 vibrions. Among the animals that received the culture containing the I-723 strain, pathological anatomical changes took place only in some of the animals and only when the dose was 10^7 vibrions. Among the animals that received the culture containing strain I-949, changes were seen with both doses, but only in isolated cases.

*Cf. "Metodicheskiye Rekomendatsii po Opredeleniyu Virulentnosti Kholernykh Vibrionov na Modeli Krolikov-Sosunkov" [Methodological Recommendations for Determining the Virulence of Cholera Vibrions Using Baby Rabbits as Models], Rostov on Don, 1979.

Among the animals that were given the culture with strain I-935, histological changes took place in the stomach in the form of hyperemia in the mucosa and submucosa, areas of loosening in the end sections of glands, and the presence of numerous vibrions on the surface of the mucous membrane or free in the stomach cavity (in the middle of sloughed epithelium).

In the duodenum at first there was moderate edema in the stroma of separate fibers; in places there was vacuolization of the epithelial membrane; infiltration of the stroma by single pseudo-eonsinophiles was noted in places; there was subsequent hyperemia of the mucosa and submucosa, loosening and tearing away of the end sections of some fibers and an abundance of vibrions in the lumen of the intestine (in the middle of sloughed epithelium and torn fibers), on the surface of the fibers, in the stroma of various fibers, and in the follicles and submucosal membrane.

In the jejunum and ileum we found hyperemia and edema in the mucosa and submucosa, moderate infiltration by pseudo-eosinophiles with the latter in epithelial cells, vacuolization and granular dystrophy of the epithelium, loosening and tearing of the epithelium, exudate consisting of a homogeneous oxyphilic mass in the intestinal lumen along with sloughed epithelium, pseudo-eosinophiles and a small number of vibrions (on the surface of the fibers, in the epithelium, and in the intestinal lumen). During the agonal period of the disease and in the dead animals we found hyperemia and edema in the submucosal membrane, loosening, necrosis, and tearing of the epithelium and end sections of fibers, and an abundance of vibrions in the lumen, in necrotic sections of the mucosa, and in the follicles and stroma of individual fibers.

In the large intestine we found hyperemia and edema in the mucosa and submucosa, infiltration of these layers by pseudo-eosinophiles; in places we found pseudo-eosinophiles in the epithelium, a few vibrions on the surfaces of folds; and in some animals, we found intestinal contents with an oxyphilic mass, sloughed epithelium, vibrions, and pseudo-eosinophiles. In the agonal period of the disease and in the dead animals in addition to these changes we also found granular dystrophy and areas of vacuolization in the epithelium, loosening, tearing, and necrosis of epithelium in the folds, infiltration of the mucosa and submucosa by lymphocytes, and an abundance of vibrions (in the lumen, on the surface and in the epithelium of folds, and in follicles).

In the liver we found granular dystrophy of parenchymal cells and infiltration by eonsinophiles (mixed in with elements usually found in baby rabbits when there is local infiltration of the liver by myeloid elements).

In the kidneys we found granular dystrophy of the epithelium of the proximal convolute tubules, with the primary dystrophy occurring near the medullar zone; there was excess blood in the capillaries, veins, and in some glomeruli.

During the agonal period of the disease and in the dead animals we found in the myocardium irregular granular dystrophy of the myocardial cells.

In the spleen we found infiltration (diffuse or localized) by red pulp and sometimes in the ends of follicles there was infiltration by pseudo-eosinophiles; there was excess blood in the sinuses in some cases.

In animals that were given the culture containing strain I-723, a similar histological picture was characterized by a relatively low level of edema in the stroma of fibers and vacuolization of the epithelium covering the fibers. There was also more significant loosening and tearing of the epithelium on the end sections of the fibers, as well as accumulation of vibrions on the surface of the fibers and deeper in the epithelial layer both where it was loosening and where no structural damage was evident. In the agonal period of the disease and in the dead animals the intestinal infection was generally of the same character and expressed to the same extent as that found in animals that had been given the culture containing strain I-935; it differed however, in that there was significant hyperemia in the mucosa and submucosa with hemorrhaging, and loosening, tearing, and necrosis of the fibers, often in the form of marked destruction of the mucosa, usually with an abundance of vibrions and with evidence of vibrions in the epithelium and stroma of the damaged fibers.

In animals that were given the culture containing strain I-949, of interest were the marked hyperemia in the mucosa and submucosa, hemorrhaging, and fairly deep destruction of the mucosa with an abundance of vibrions penetrating the damaged areas; these effects were found only during the agonal period and in animals that died. Significant accumulations of vibrions were found in the stomachs of animals in this group; they were located on the surface of the mucosa and deeper in the mucosa with signs of loosening. There were also areas of the mucosa that were affected by hyperemia, infiltration by lymphoidal and segmental nuclear elements, and an absence of superficial layers and disorganization of glands.

In other organs we found changes similar to those that occurred in the animals in the first two groups.

In a bacteriological study of material from the animals we isolated vibrions from various parts of the gastro-intestinal tract and in some of the animals (primarily those that died), we isolated vibrions from the bile and blood.

The data that we have presented show that intra-intestinal introduction into baby rabbits of strains of Vibrio eltor isolated from objects in the environment results in acute inflammatory damage to the gastro-intestinal tract with characteristic pathomorphological changes not only in the small intestine, where the microbial suspension was introduced, but also in the stomach, the duodenum, and the large intestine. The relationship between the pathomorphological changes that we found and the peculiar features of a given strain is characterized by the fact that when a strain has a more pronounced capacity to produce an enteropathogenic effect in baby rabbits, there are more pronounced signs of edema in the intestinal wall (in the mucosa and submucosa) and there is more marked vacuolization in the epithelium of the fibers, usually with a higher or lower content of vibrions in the intestine. When a strain has a lower enteropathogenic capacity (this includes the avirulent strain) this component of inflammatory damage in the gastro-intestinal tract is less pronounced or is absent altogether; the predominant changes are hyperemia, hemorrhaging, alterative-destructive changes, often with an abundance of vibrions and with vibrions appearing in the intestinal wall.

Of special interest is the fact that the stomach and duodenum are involved, which is apparently tied to the retrograde appearance of the vibrion in these areas and to the subsequent massive accumulation of the vibrion deep in the mucosa. The latter event gives us reason to belive that the vibrion is quite enterotropic. Also, small accumulations of vibrions in apparently undamaged epithelium of the mucosa and in intercellular junctions of the epithelium allow us to assume that the vibrion is capable of invasion.

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USING DIALYSIS CULTURE METHOD TO OBTAIN DIFFUSING FRACTIONS OF PLAGUE MICROBE

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[Article by N. G. Ponomarev, Yu. V. Brandzishevskiy, and O. M. Kosmayenko (Saratov)]

[Text] Dialysis cultivation has substantial advantages over growing bacterial cultures in dense and fluid nutrient media.

A number of papers have been published on using this method to obtain protective antigens of the anthrax agent, as well as botulin, staphylococcal, diphtheria, and tetanus toxins (Gladstone, 1948; Herold et al., 1967; Lin, 1954; Mueller, 1940; Sterne et al., 1951).

The aim of the present study was to obtain diffusing antigens of the plague microbe using the method of growing cultures on semipermeable membranes, and to study the quantitative and qualitative composition of the antigens that diffuse into the medium.

The practical application of this principle of cultivation required the development of special equipment. The model device had 2 culture chambers consisting of membrane filters and a fluid nutrient medium below the filters.

In order to find the diffusing antigens we used the precipitation reaction in gel, the passive hemagglutination reaction, and the antibody neutralization reaction. To classify the antigens, we used specific sera for FI, FII, VW, and H-antigens, as well as erythrocytic antibody diagnosis for FI and lipopolysaccharide.

In the precipitation reaction with whole agglutinating plague serum, we found that the maximum number of precipitation zones in the EV strain (8) and in the K-1 strain (11) appeared with filters taken on the second day of cultivation and remained in samples taken up to the seventh day. The temperature of the culture (28° or 37°C) did not affect the number of precipitation zones recorded.

In the precipitation reaction with specific sera, FI was found to be present in diffusing fractions of the EV and K-1 strains sampled on the third and subsequent days.

In the passive hemagglutination reaction and the antibody neutralization reaction FI was detected in the diffusing fractions of both strains also on the third day in titers of 1:4 and 1:8; on the fifth and sixth day the titers increased to 1:64 and 1:128. One of the samples of the K-1 strain was an exception; it was taken on the second day and in the passive hemagglutination reaction FI was found in a titer of 1:4.

In the precipitation reaction, "mouse" toxin was detected in diffusing fractions that were sampled starting on the third day.

In the passive hemagglutination reaction and the antibody neutralization reaction lipopolysaccharide appeared in the diffusing fractions on the third day after the culture was started in titers of 1:8-1:16; by the sixth or seventh day the titers had risen to 1:164-1:256.

In the precipitation reaction the VW- and H-antigens were not detected in the diffusing fractions at any point in the study.

In tests on white mice we determined the toxicity of diffusing fractions through intra-abdominal introduction of fractions taken on the fifth day of the culture. The LD_{50} of the fractions of the EV strain was 0.05 mg and for the K-1 strain, it was 0.02 mg (of protein).

In tests on guinea pigs we determined the immunological activity of the diffusing fractions of the EV and K-1 strains taken on the fifth day of the culture. Twenty-one days after a single subcutaneous injection of diffusing fractions containing 1 mg (by protein), 60-80 percent of the animals survived contamination by 20 Dcl of the virulent 231 strain of the plague microbe.

This method of dialysis cultivation can be used to obtain diffusing sterile fractions of the plague microbe over the entire culture period, which is especially valuable when working with virulent strains. In the future this method can be used successfully to work out under the dynamics of optimal conditions the synthesis of necessary antigens of the plague microbe, and to study the effect of widely varying groups of substances on the viability of a population by adding them to the nutrient medium.

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SOME CHARACTERISTICS OF METABOLISM OF PLAGUE MICROBE

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[Article by V. G. Mayskiy (Stavropol)]

[Text] Some specific achievements have been made in the study of the metabolism of the plague microbe, which are reflected in a number of monographs (Martinevskiy, 1969; Domaradskiy et al., 1974). However, it has become possible only recently to solve many of the questions concerning the metabolism of this agent; the widespread application of contemporary bacteriological, biochemical, and genetic research methods is responsible for this. The present paper on the whole contains a description of a study of the characteristics of the metabolism of the plague agent that was done at the Biochemistry of the Plague Microbe Laboratory at the Antiplague Scientific Research Institute of the Caucasus and Transcaucasus.

In studying the effect of several glycine-containing di- and tri-peptides on the inclusion of ^{14}C-glycine in the proteins of the plague microbe, we determined their competitive and specific inhibition of this inclusion; in a number of cases this inhibition was quite high (up to 87 percent), but the growth of bacteria and protein synthesis within the bacteria did not slow down. The results we obtained gave us reason to suppose that some low-molecular peptides can be used in the biosynthesis of protein better than free amino acids. Indeed, when growing the plague microbe in complete nutrient media containing peptides, we observed insignificant inclusion of ^{14}C-methionine and ^{14}C-phenylalanine, which are "essential" amino acids. Poor utilization of free amino acids by the plague microbe in complete nutrient media was also confirmed by chromatographic methods. At the same time, inclusion of these amino acids was high when plague bacteria were grown in a peptide-free synthetic medium.

The results we obtained allow us to re-examine the traditional perception of free amino acids as the source of nitrogen for protein used most by the plague microbe. On the basis of data in the literature on the content of proteins and their precursors in the blood and tissues of rodents with plague (Domaradskiy, 1966), we can assume that there are changes in the content and polymer aspects of proteins in the presence of plague infection. A possible increase in the concentration of easily assimilated peptides in the blood and tissues of an animal with plague will help speed up the multiplication of plague bacteria,

that is, in the final analysis it will promote the development of the infection process.

Many investigators tie the extent to which the potential pathogenicity of the bacteria is realized to the specific aspects of biological oxidation of the bacteria. The energy system, specifically the respiratory chain of the microorganisms, is extremely labile (Gel'man et al., 1966). One can judge the activity of the respiratory chain by the intensity at which the nonphysiological hydrogen acceptors are used, including 2, 3, 5-triphenyltetrazolium chloride (TTC). Compared to saprophytes (intestinal bacilli and common proteus), the use of TTC by the plague microbe was quite low. As a result of the fact that TTC directs the respiratory chain along a false course, and in the final analysis leads to retardation in the growth of the bacteria, this retardation is especially significant for saprophytes that have a high level of TTC-dehydrogenase activity. In our experiments we observed a 70-85 percent inhibition of the growth of microflora taken from a rinse of objects in the environment after 2 days of growth; under these same conditions there was only a 4-14 percent inhibition of the growth of the plague agent. The fact that the plague microbe uses the nonphysiological hydrogen acceptor much later and to a lesser extent provides certain selective advantages in cultures in nutrient media containing TTC.

In a study of the exchange of purine compounds, it was shown that with the plague microbe, unlike the pseudotuberculosis agent in rodents, ^{14}C-adenine is not included in the guanine of polynucleotides when histidine is present in the nutrient medium. Of the 3 possible pathways for turning exogenous adenine into the guanine of nucleic acids in the plague microbe, there is only one that involves the biosynthesis of histidine. In the plague microbe, unlike the pseudotuberculosis agent in rodents, the pathway that requires deamination of adenosine before inosine is missing. We studied the activity of adenosine desaminase and the optimal conditions for its manifestation in the pseudotuberculosis agent in rodents, however efforts to induce this activity in the plague agent were unsuccessful. On the basis of this difference, we developed a reliable and rapid (2-hour) method for differentiating plague and pseudotuberculosis microbes in terms of adenosine desaminase.

We studied the metabolism of pyrimidine bases and nucleosides in plague and pseudotuberculosis microbes. We found another feature that distinguishes the plague microbe from the pseudotuberculosis agent in rodents and enterobacteria: the plague agent is not capable of assimilating thymine, that is, it is lacking the enzyme thymidinephosphorylase. From this it follows that using thymine for specific marking of the DNA of the plague microbe is not possible in practice and obtaining mutants that are dependent only on thymine is not promising. On the other hand, ^{14}C-thymidine, which does not break down in the presence of thymidine phosphorylase, remains for a long time in the DNA of the plague microbe and thus is a highly effective marker.

The defects that have been found in the plague microbe regarding the use of exogenous adenine and thymine turned out to be inherent in all the strains we studied, regardless of differences in terms of their biological properties, virulence, and the time and place they were isolated (the Caucasus, Mongolia, China, India, Central Asia, Trans-Baykal, etc.)

As a result of the research we did, we revealed a number of peculiarities in the metabolism of the plague microbe. The deficiency of a number of enzymes in the plague bacteria compared to other microorganisms, weaker activity of other enzymes, and the resulting limited possibilities for utilizing a number of compounds, including possibly substances that participate in the macroorganism's defense responses, may under certain conditions promote the survival and multiplication of the microbe in the internal conditions of the macroorgansim; that is, they may contribute to an important biological property of the plague microbe--its virulence.

On the basis of the known similarity between the plague and pseudotuberculosis microbes in terms of many of their biochemical and antigenic properties, and on the basis of recent experiments on the molecular hybridization of their DNA, we can view these bacteria as subtypes of single type (Bercovier, Mollaret et al., 1980). However, the genetically determined biochemical differences between the plague and pseudotuberculosis microbes indicate that in the plague microbe dozens, if not hundreds, of genes that are characteristic of the pseudotuberculosis agent in rodents, are absent or are repressed. On the basis of the presence of already known considerable and significant differences in the genotype of the plague microbe and the pseudotuberculosis microbe, they can hardly be considered subtypes rather than independent types of bacteria.

Therefore, the possibilities for the plague microbe to realize the majority of the biochemical properties studied are more limited than those of a number of other microorganisms; this, on the one hand, can be reflected to some extent in a selective approach to types of natural carriers and transmitters of this infection; and, on the other hand, this can in a number of cases help plague bacteria become more resistant to inhibiting and therapeutic preparations.

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BEHAVIOR OF MONOAUXOTROPHIC MUTANTS OF VIBRIO CHOLERAE IN MIXED CULTURES WITH ORIGINAL STRAIN

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[Article by P. Ya. Ognevskiy (Alma-Ata)]

[Text] It is well known that under the conditions of the external environment under the influence of various agents, diverse mutations occur, including mutations involving auxotrophy. The frequency of these mutations is low, but due to a higher rate of multiplication among several types of mutants, they have certain advantages in the competitive struggle for survival.

As a result of these effects in microorganisms, including in strains of V. cholerae, there can be changes in the morphology of colonies and cells, biochemical activity, antigenic structure, nutrient needs, and so forth. We studied the effect of auxotrophic mutants of V. cholerae on the rate of growth of the original strain when they were cultured together.

In the experiment we used a strain of V. cholerae of the eltor biotype and with serotype Inab A-473, and 6 monoauxotrophs, obtained under the influence of nitrosoguanidine, with varying nutrient requirements: A-473-25-2 is histidine-dependent; A-473-17-1 is isoleucine-dependent; A-473-33-3 is arginine-dependent; A-473-56-15 is leucine-dependent; A-473-32-11 is inosite-dependent; and A-473-63-16 is thiamine-dependent.

We used 18-20 hour agar cultures of cholera vibrions. The suspensions were prepared according to the standard of the Medical Biological Preparations Control Institute imeni L. A. Tarasevich in a physiological NaCl solution with a concentration of microbial cells of $1 \cdot 10^9$ per ml. For the combined culture we introduced 0.1 ml of the dissolved suspension into 5.0 ml of minimum medium A to which the corresponding amino acid had been added in a concentration of 0.03 µg/ml. The concentration of microbial cells in the medium was 200/ml. The mixed cultures were incubated at 37°C and daily samples were taken to calculate the number of live cells of the mutant and of the original strain that had grown in Marten's agar. Before colonization the broth cultures were diluted 10-fold in a physiological NaCl solution in order to grow 50-100 isolated colonies on an plate of agar. The cultures were incubated at 37°C for 18-20 hours and then using the imprint method the colonies were transferred to dishes containing minimum medium A with 0.1 percent glucose. The results were

computed after 18-20 hours of incubation by comparing the original dish with the cultures that had grown in the minimum medium. The colonies that were not grown in the minimum medium were classified as auxotrophic mutants. The number of colonies of the original strain was determined by finding the difference between the quantity of colonies that had grown in Marten's agar and in the dish containing the minimum medium.

During the first 24 hours of combined cultivation the number of live cells of histidine-dependent and isoleucine-dependent strain exceeded the number of cells of the original strain by a factor of 50-100 and totalled $1.1 \cdot 10^7$ and $2 \cdot 10^6$ microbial cells/ml, respectively. Over the course of the observation period (30 days), the concentration of live mutant cells exceeded the number of cells of the original strain by a significant amount.

In the combined cultivation of arginine-, leucine-, inosite-, and thiamine-dependent auxotrophs and a prototroph, after 24 hours of incubation the number of live mutant cells was $2 \cdot 10^5$, $2 \cdot 10^4$, $1.8 \cdot 10^5$, and $6 \cdot 10^4$ microbial cells/ml, respectively. The concentration of cells of the original strain was $2 \cdot 10^7$, $2 \cdot 10^6$, $4 \cdot 10^6$, and $1.1 \cdot 10^5$ microbial cells/ml; that is, the number of live mutant cells dropped to one-one thousandth, one-one hundredth, one-twentieth, and one-half of the previous level. On the fourth day the cells of the leucine-, inosite-, and thiamine-dependent mutants died off completely. After 15 days the cells of the arginine-dependent mutant died off and there were high concentrations of cells of the original strain. In the control test tubes we planted mutant cells over the entire course of the observation period.

The mutant cells died off apparently because of an unidentified product of metabolism of the cells of the original strain.

Thus, the rate of growth of histidine- and isoleucine-dependent auxotrophic mutants of the cholera vibrion was significantly lower than that of the original strain, while the leucine-, inosite-, and thiamine-dependent mutants fell behind the rate of growth of the original strain and it was not possible to isolate them on the fourth day of incubation.

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USE OF MILK-AND-SALINE AGAR TO OBTAIN CAPSULES IN BACILLUS ANTHRACIS

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[Article by A. I. Kalinovskiy, A. V. Rodzikovskiy, and Yu. I. Sorkin (Irkutsk)]

[Text] One of the taxonomic signs distinguishing anthrax agents from other representatives of the Bacillus genus is the ability of B. anthracis to form a capsule.

Since the study of capsule formation in vivo involves certain difficulties, an in vitro method is used more frequently. With this aim various elective media are used, the majority of which have as a necessary component native bovine or equine serum in a relatively high concentration (media from the Medical Biological Preparations Control Institute imeni L. A. Tarasevich, Lefler's medium, serum agar, and serum broth).

Today the study of capsule formation in vitro is done in media that contain blood, serum, egg albumin, brain tissue, and bicarbonate in the presence of 10-50 percent CO_2 at 37°C. Torn's and Booze's media are used for this purpose. All these media are quite complicated to prepare and some of them also have numerous components.

The most distinct results in capsule formation are obtained when strains of the anthrax agent are cultured in a fluid or coagulated serum (Lefler's medium), and in a 40 percent serum agar. Obtaining, storing, and using blood serum from cattle or horse involves certain material and technical difficulties.

We have developed a method for obtaining capsules in B. anthracis in a milk and saline agar.

The available literature does not contain any data on using a milk-and-saline agar to determine capsule formation in strains of B. anthracis.

We prepared the milk and saline agar according to the generally accepted method that involves adding 5 percent sterile milk treated with chloroform to a melted and cooled 3 percent saline agar. We used 27 virulent strains and 2 avirulent strains of B. anthracis. The virulent strains were isolated from humans and animals in Siberia and the Far East. For the avirulent strains we used the STI-1 vaccine strain and the I-47 strain, isolated from experiments on foxes in

the Tuva ASSR. We also studied capsule formation in B. cereus var. mycoides 412, B. anthracoides 365, B. subtilis 501, B. mesentericus 61, and B. megaterium 512, obtained from the All-Union Collection of Microorganisms and Algae. To create optimal culture conditions for the strains we used a CO_2 incubator from the "Labomed" company (FRG), in which the prescribed 10 percent carbon dioxide concentration and a temperature of 37°C were maintained automatically. The presence of capsules in the preparations was determined by staining them with Rebiger's stain and Burry's ink.

The results of the research showed that after 18-20 hours of incubation, a well-defined capsule appeared in all the virulent strains, especially in those preparations stained with ink. We noted that in the milk and saline agar capsulization in single bacilli and in bacilli linked in a chain was expressed more intensively and at earlier stages of incubation than when these same strains were grown in 20 and 40 percent serum agar and in whole equine serum.

The avirulent strains of B. anthracis I-47 and STI-1 and the B. cereus var. mycoide 412, B. anthracoides 365, B. subtilis 501, B. mesentericus 61, and B. megaterium 512 strains cultured in whole serum, serum agars, and the milk and saline agar under the same experimental conditions as the virulent strains, did not form capsules. The observation period was from 18 hours to 3 days.

Therefore, in order to determine capsule formation in anthrax agents in vitro, we can recommend a milk and saline agar. This medium is more economical and simpler to prepare than media that are commonly used and contain significant quantities of blood serum from animals.

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MINIMUM SYNTHETIC NUTRIENT MEDIA FOR BRUCELLAE

Moscow ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII in Russian No 11, 1983 p 112

[Article by D. N. Donskoy, A. F. Pinigin, Ye. P. Golubinskiy, and S. P. Merinov (Irkutsk)]

[Text] Interest has developed recently in clarifying the nature of the nutrient requirements of pathogenic microorganisms, including brucellosis agents. Numerous variants of synthetic nutrient media have been suggested for growing brucellae. The most well-known modifications are those described by Kondrat'yeva (1968), Merinov et al. (1980), Koser et al. (1941), McCullough and Dick (1943), McCullough et al. (1947), Gerchardt and Wilson (1948), Polter (1950), and Rode et al. (1950). The majority of synthetic media that have been suggested were developed with the aim of obtaining a high yield of microbes and only a few were developed as minimum media. In addition to the mineral base, the well-known minimum media contained a fairly large quantity of additional growth factors. For example, the number of amino acids in them ranged from 2 to 6 and the number of vitamins from 2 to 5. The minimum nutrient media most often contained the amino acids: l-glutamic acid, l-arginine, l-histidine, l-lysine, dl-methionine, l-cysteine, l-cystine; and the vitamins: thiamine-HCl, 7-nicotinic acid, 6-pantothenate, biotin, and riboflavin. When developing the minimum media, large culture doses were used as a rule (an automatic dispenser or special plates were used to plant the material).

Taking all this into account, we tried to design a minimum synthetic nutrient medium that would provide growth of brucellae when single microbes were planted. This type of medium is needed to obtain auxotrophic mutants and to carry out selection in other genetic research.

In designing the medium, we used as a base a saline composition that included monosubstituted potassium phosphate, sodium chloride, sodium sulfide, and ammonium chloride. We added sodium pyruvate to the medium to meet the energy requirements of the brucellae. We initially added 18 amino acids and 10 vitamins to the medium as additional growth factors. By decreasing the quantity of amino acids in the medium and grouping them in various combinations, we concluded that the brucella cultures can grow only if methionine is present in the medium. Then we successively removed the vitamins from the medium. Three vitamins turned out to be essential: nicotinic acid,

cocarboxylase, and biotin. To give density to the medium we added 2 percent agar (Difco's agar).

To test the medium we used primarily reference strains of brucellae described in the chart developed by the Subcommittee for the Taxonomy of Brucellae of the FAO/WHO. To place the cultures in the medium we used the pipette method for planting a bacterial suspension. The strains were planted in quantities of 15,000, 1500, and 150 microbial cells. The cultures were kept in a thermostatically controlled chamber at 37°C. The results were evaluated after 18 days. The experiments were repeated 3 times and conclusions were made on the basis of the summarized data.

The growth of brucella colonies appeared between the fourth and seventh days and at first, as a rule, in the form of very small (dew-like) colonies. On the fifth and sixth days colonies formed that were about 1 mm in diameter, and between the eighth and eighteenth days they had reached 1.1-2.0 mm or larger. When the planting doses were 150 and 1500 microbial cells, the colonies were somewhat larger than when the dose was 15,000.

To determine the growing properties of the medium, we planted strains of Brucella melitensis 16 M, B. abortus 544, and B. suis 1330 alongside the other strains in a minimum medium and a rich (glucose-glycerine Marten's agar) medium, in quantities of 15, 150, and 1500 microbial cells. It turned out that the effectiveness of the growth of brucellae in the minimum medium was 85 percent of the control growth in the rich medium.

Thus, the results of our study to determine the nutrient requirements of brucellae made it possible to design a minimum synthetic nutrient medium that contains monosubstituted potassium phosphate, sodium chloride, sodium sulfide, ammonium chloride, sodium pyruvate, methionine, nicotinic acid, cocarboxylase, and biotin.

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INCIDENCE OF YERSINIOSES IN KAZAKHSTAN

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[Article by V. M. Stepanov, L. S. Bezrukova, N. P. Arkhangel'skaya, T. P. Kudinova, S. N. Kalimbekov, T. I. Dmitrovskaya, and A. M. Dmitrovskiy (Alma-Ata)]

[Text] In our country a great deal of attention is being given to the role of yersinioses in human pathology. In light of the fact that there is practically no information at all on the incidence of yersinioses in Kazakhstan, we made the first extensive survey of yersinioses in four oblasts of Kazakhstan, in Alma-Ata, and in a limited region of the Kara-Kalpak ASSR.

A total of 2075 humans, 482 agricultural animals, and 12,134 rodents were subjected to a bacteriological examinatin. We found a significant incidence of pseudotuberculosis and intestinal yersiniosis.

After a bacteriological examination, Yersinia pseudotuberculosis was isolated in 8.5 percent of the patients with an unclear diagnosis, and Y. enterocolitica was isolated in 9.2 percent of the patients with an unclear diagnosis. A serological study of the patients for pseudotuberculosis made it possible to diagnose this infection in 17.5 percent of those examined.

A study of the characteristics of the course of the diseases made it possible to establish the particular aspects of pseudotuberculosis and intestinal yersiniosis under conditions in Kazakhstan and to develop a classification of clinical forms applicable to local conditions, in addition to a system for treating these illnesses.

With the aim of studying the long-term outcome of yersinioses, 169 of the patients we examined during an acute stage were observed on an out-patient basis for a period between 1 month and 2 years following the illness. In 21 of them (12.4 percent) we found Y. pseudotuberculosis and Y. enterocolitica. The agent was isolated from 12 individuals who had had pseudotuberculosis (14.2 percent) and from 9 who had had intestinal yersinosis (10.6 percent). We found antibodies in diagnostic titers (from 1:320 to 1:2500) in 11 of the people who had had pseudotuberculosis (13.1 percent) and in 6 who had had intestinal yersinosis (7.1 percent).

A comparison of the time periods at which the agent was isolated and the status of intestinal microflora in the individuals who had had yersinosis allowed us to establish a correlation between the length of time that yersinia bacteria can be found and the extent to which dysbacteriosis is evident in the intestine. Apparently, dysbacteriosis is one of the factors that contributes to prolonged retention of the agent in the organism.

In a bacteriological study of rodents in Alma-Ata we found a high level of infection (3.6 percent were infected with pseudotuberculosis and 9 percent were infected with intestinal yersiniosis).

Among wild rodents (primarily large peschankas), 1.7 percent of those studied in Mangyshlak Oblast were infected with pseudotuberculosis and 0.8 percent of those studied in Kara-Kalpak ASSR were infected with pseudotuberculosis. In other oblasts we did not find rodents infected with pseudotuberculosis, but we did find animals with specific antibodies in Taldy-Kurgan Oblast; in various areas they accounted for 0.2 to 1 percent of the animals we examined.

Intestinal yersiniosis is more widespread among rodents than pseudotuberculosis: it was found everywhere that we did research on the infection.

We found simultaneous epizootics of plague and pseudotuberculosis in the same population of large sand rats.

In light of this, it is necessary to study the question of the influence of the circulation of the pseudotuberculosis microbe on the course of the plague epizootic (the formation of a layer of immunity, a change in the organism's sensitivity, the possibility of forming on this basis prolonged forms, aggravated forms, and so forth).

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FINDING ANTIGENS OF FRANCISELLA TULARENSIS USING IMMUNO-ENZYME METHOD

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[Article by V. M. Polyachenko, S. P. Merinov, Ye. P. Golubinskiy, N. Zh. Gracheva, A. M. Kokha, and L. V. Merinova (Irkutsk)]

[Text] Today the immuno-enzyme method (ELISA) [enzyme-linked immunosorbent assay] is playing an increasingly important role in the diagnosis of both infectious and noninfectious diseases. In infection pathology it is used primarily to find specific antibodies and less frequently, to identify certain bacterial antigens, such as those of the plague microbe, cholera vibrion, salmonellae, and staphylococcus. The advantages of this method include a high degree of sensitivity (usually higher than the sensitivity of the classical immunological reactions, such as the agglutination reactions, the indirect hemagglutination reaction, etc.), specificity, rapid identification, and ease of performance.

The immuno-enzyme method to determine the titer of specific antibodies in serum taken from people who have had tularemia and in serum from animals who have been infected experimentally with F. tularensis, was first proposed by Ca-l·.i et al. (1979). The available literature does not contain any data on the application of this method to determine antigens of the tularemia microbe. At the same time, the development of a rapid, highly sensitive and specific method for identifying the tularemia antigen is of great interest from the standpoint of doing epizootological research on tularemia.

N. G. Olsuf'yev (1975) reported that the neutralization reaction of antibodies with an antigenic erythrocytic diagnostic test, used to study material from corpses, rodents nests, and bird droppings, makes it possible to find the tularemia microbe when its concentration is 1 million per 1 ml (according to the standard set by the Medical Biological Preparations Control Institute imeni L. A. Tarasevich); and the passive hemagglutination reaction with an antibody erythrocytic diagnostic test makes it possible to find the tularemia antigen obtained by Bouaven's method, in units as small as one-ten thousandth of a microgram.

We have developed a variation of the immuno-enzyme method for a quantitative determination of the tularemia antigen. The reaction was set up in disposable polystyrene microplanchets produced at the Leningrad Medical Polymers Plant. To sensitize the alveoli we used immunoglobulin fractions obtained from

commercial equine anti-tularemia sera produced at the Irkutsk Antiplague Institute (the titer in the agglutination reaction was 1:3200 and in the indirect hemagglutination reaction it was 1:500,000). The immunoglobulins were isolated by means of fractionation of the sera using ammonium sulfate, caprylic acid, or polyethylene glycol-6000. For the tularemia antigen we used a water-soluble lipopolysaccharide obtained from the vaccine strain of F. tularensis No 15 by means of the method described by Westphal and Lüderitz (1952), and whole cells of the same strain, killed by a 1 percent formalin solution. Conjugates of the immunoglobulin fraction from equine anti-tularemia sera with peroxidase from horseradish (the "Serva" company, FRG) were prepared according to the method described by Nakane (1974).

As a result of the preliminary experiments for setting up the reaction, we chose a sandwich modification of the immuno-enzyme method. To select the optimal conditions for carrying out the reaction, we sensitized the alveoli of the microplanchet with immunoglobulin fractions in concentrations ranging from 20 to 100 µg of protein per 1 ml in a 0.05 M carbonate buffer with a pH of 9.6. The sensitization period varied from 30 minutes to 3 hours at 37°C and from 1 to 18 hours at room temperature. With the same goal we brought the lipopolysaccharide or whole cells of the tularemia microbe in contact with sensitized alveoli for 1 to 3 hours at room temperature and at 37°C. We treated the antibody-antigen complex with the immunoglobulin-peroxidase conjugate for 1-2 hours.

Finally, we selected the following course for determining F. tularensis antigens. The microplanchet alveoli were first sensitized by immunoglobulins (50µg of protein per 1 ml, for 1 hour at 37°C), then thoroughly rinsed with a buffered physiological solution (a 0.85 percent NaCl solution with a 0.15 M phosphate buffer and a pH of 7.4) that contained 0.05 percent Tween-20 and 0.1 percent bovine serum albumin; they were air dried and then filled with 100 µl of lipopolysaccharide or whole cells in varying concentrations and incubated at 37°C. The control alveoli contained only a diluting solution (0.05 M carbonate buffer with a pH of 9.6). The antigens that did not form complexes were washed from the alveoli 3 times with a buffered physiological solution; then each alveolus was filled with 100 µl of the immuno-peroxidase conjugate diluted in a 0.04 M phosphate buffer with a pH of 7.4 up to a concentration corresponding to its working titer, and they were then incubated for 1 hour at 37°C. After a thorough rinsing, 100 µl of a 0.01 percent solution of o-phenylendiamine containing 0.03 percent H_2O_2 was introduced into the alveoli. The results were evaluated visually on the basis of a brown or orange stain developing after a 20-25 minute exposure and interrupting the reaction with 8 n. H_2SO_4, under the condition that there was no staining in the control alveoli.

If one does not take into account the time spent on sensitizing the microplanchets, which can be done ahead of time, setting up and evaluating the reaction takes on the average no longer than 3 hours.

As a result of the experiments we performed, we established that the concentrations of the tularemia microbe lipopolysaccharide found consistently range between 1 and 3 µg/ml, and the whole cells of F. tularensis are found in concentrations of $6 \cdot 10^5$ per 1 ml. This sensitivity does not exceed the sensitivity obtained in ordinary hemagglutination reactions. We still believe,

however, that further improvements in the immuno-enzyme method in the direction of finding more active immunosorbents and obtaining purer and specifically active immunoglobulin preparations, highly active immuno-enzyme conjugates, optimization of the reaction conditions, and so forth, will make it possible to increase markedly the sensitivity of determining antigens of the tularemia microbe.

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DEHYDROGENASE ACTIVITY OF PLAGUE VACCINE STRAINS AS INDIRECT INDICATOR OF IMMUNOGENICITY

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[Article by A. I. Tinker, L. I. Zarevina, and N. M. Khar'kova (Stavropol)]

[Text] It is known that some properties of the plague vaccine strains can serve as indirect indicators of their immunogenicity. These include "latent" virulence, the ability to multiply and take hold in organs and tissues of laboratory animals, and so on. The methods for determining these properties in the microbes are not complicated, but they are labor-intensive, time-consuming, and relatively costly. We were interested in the possibility of using biochemical tests to evaluate the immunogenicity of vaccine strains. Khar'kova et al. (1976) reported that the K-1 strain of the plague microbe had a higher level of dehydrogenase activity than the EV vaccine strain. It is well known that it is also more immunogenic in guinea pigs. Drozovskaya et al. (1977) noted that plague vaccines with a high degree of immunogenicity have active oxidative metabolism. The authors studied the triphenyltetrazolium chloride-reductase activity in terms of several metabolites of the Krebs cycle. We set ourselves the task of studying the dehydrogenase activity of full-strength plague vaccine strains and strains with various defects in their antigenic structure, and with resulting variations in immunogenicity.

In the experiments we used the K-1, EV, EVFI-, and Otten's plague vaccine strains. The K-1 and EV strains are complete in antigenic terms; the EVFI- strain is missing fraction I; and Otten's strain is missing the VW-antigens. The results of our additional research showed that the toxigenicity of the EVFI- and Otten's strains was approximately one-three hundredth to one-four hundredth that of the K-1 and EV strains.

The cultures used in the experiment differed in terms of their immunogenic activity in guinea pigs and mice. According to the data we obtained in studying the intensity of immunity and immunogenicity in experiments on both types of biological test animals inoculated subcutaneously and intra-abdominally with subsequent infection by a virulent plague microbe culture according to the generally accepted methods, the K-1 strain was the most immunogenic in guinea pigs; the EV strain had a significantly lower immunogenicity, with Otten's strain and the EVFI- strain following. In white

mice the immunogenicity of the K-1 and EV strains was identical; Otten's strain was mildly immunogenic and the EVFI- strain was practically nonimmunogenic.

We determined the dehydrogenase activity according to the method described by Kuna and Abood (1949) as modified by Mironova and Merinov (1972). For substrates we used 20 different amino acids: dl-aspartic acid, L-arginine, l-asparagine, dl-alanine, dl-valine, glycine, l-glutamic acid, l-histidine, l-glutamine, l-isoleucine, dl-leucine, dl-lysine, dl-methionine, l-ornithine, l-proline, dl-serine, dl-threonine, dl-citrulline, l-cystine, and dl-ß-phenyl-L-alanine. The incubation mixture was kept in 1 ml of a 20 billion suspension of bacteria grown at 28° in Hottinger's agar for 48 hours and rinsed 3 times from the nutrient medium with a refrigerated physiological solution of sodium chloride. We determined the extent of dehydrogenase activity on the basis of optic density. The experiments were repeated 10 times and the results were analyzed using variant statistics.

The results of the experiments showed that all the strains studied dehydrogenated the amino acids used in the experiments. The intensity of dehydrogenation of the amino acids can be divided into 3 groups. In the first group the extinction indicator did not exceed 0.05; in the second group it ranged from 0.06 to 0.15; and in the third group it was between 0.16 and 0.25. For the K-1 strain, there were 14 amino acids in the second group and 5 in the third group (l-glutamic acid, dl-ornithine, l-glutamine, dl-serine, and dl-threonine). For the EV strain, there was 1 amino acid in the first group; 15 amino acids in the second group; and 3 amino acids in the third group (l-glutamic acid, dl-serine, and l-isoleucine). A diametrically opposite distribution of the intensity of dehydrogenation is observed when the EVFI- and Otten's strains are used. For the EVFI- strain there are 12 amino acids in the first group; 6 in the second group; and 2 in the third group (l-glutamic acid and dl-serine). For Otten's strain there are 14 amino acids in the first group and 5 in the second group. Consequently, there is a difference in the dehydrogenation of amino acids in the strains we studied. According to our evaluation of the intensity of dehydrogenation of the substrates, the strains can be arranged in the following order: K-1, EV, EVFI-, and Otten's strain. The K-1, EV, and EVFI- strains were the most active in dehydrogenating 6 amino acids. Of these, 2 amino acids (l-glutamic acid and dl-serine) were common to all the strains. These amino acids, however, are not among those that are essential for the growth of these strains in synthetic media.

An analysis of the intensity of dehydrogenation of the given substrates by the strains being studied showed that the K-1 strain breaks down 4 amino acids (glycine, ornithine, citrulline, and leucine) to a significantly greater degree than does the EV strain. Similar results were obtained when we compared the EV strain with the EVFI- and Otten's strains.

Thus, we found a correlation beteen the immunogenicity of plague vaccine strains and their dehydrogenase activity on amino acids. Strains that were complete in antigenic terms and highly immunogenic had the highest degree of dehydrogenase activity on amino acids. Strains that were defective in terms of a number of antigens (fraction I, VW, toxin) and had a lower level of immunogenicity in guinea pigs and white mice, had a level of enzymatic activity on amino acids that was considerably lower.

Therefore, the simple, economical, and rapidly reproduced biochemical method of determining the hydrogenase activity on amino acids can be used as an additional test for making a preliminary selection of highly immunogenic strains.

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USING ENZYME-MARKED ANTIBODY REACTION IN EPIZOOTOLOGICAL SURVEYS OF NATURAL PLAGUE NIDI

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[Article by V. N. Zhuravlev, A. T. Yakovlev, V. S. Rybkin, L. F. Zykin, I. I. Cherchenko, V. S. Burkin, L. S. Petrova, and F. Kh. Ibragimov (Astrakhan, Volgograd)]

[Text] Today the indirect hemagglutination reaction is used widely in epizootological surveys of natural plague nidi; it is used to determine a type-specific antigen of the plague microbe in various objects and to find antibodies in susceptible animals (Stepanov et al., 1967; Kanatov, 1969; Marin et al., 1978; etc.) However, the indirect hemagglutination reaction and its modifications are less sensitive than bacteriological and biological methods and the commercial erythrocytic diagnostic tests are not able to detect atypical (uncapsulated) strains of the plague microbe (Klassovskiy, 1975; Kuznetsov et al., 1979). Therefore, the effectiveness of epizootological surveys of natural plague nidi can be increased only by introducing new, highly sensitive serological diagnostic methods, such as the enzyme-marked antibody reaction.

The present paper contains material on using the enzyme-marked antibody reaction to find antigens of the plague agent in various objects of epizootological surveys of natural nidi.

The enzyme-marked antibody reaction was set up in a "double antibody" variation of the method described by Voller et al. (1976), which we modified; we used polystyrene planchets produced by the Leningrad Medical Polymers Plant. In the reaction we used immuno-peroxidase conjugates prepared according to the 2-stage method described by Avrameas et al. (1978). The indirect hemagglutination reaction with a commercial plague immunoglobulin diagnostic test served as the control.

In the first stage we studied the sensitivity of the enzyme-marked antibody reaction and evaluated it using different strains and antigens of the plague microbe. We established that the conjugates obtained expose in the reaction 0.5 ng of fraction I per 1 ml or $7.5 \cdot 10^{5}$ cells of Yersinia pestis per 1 ml.

With the aim of studying the possibility of using the enzyme-marked antibody reaction in research on wild rodents, the primary carriers of plague in the Volga-Ural steppe and in sandy nidi, we examined 374 small gophers, diurnal and crested peschankas, artificially infected with virulent strains of Y. pestis. In a bacteriological examination of suspensions of their internal organs, we found the plague agent in 60.7 percent of the samples. With the enzyme-marked antibody reaction we found antigens of the plague microbe in 47.9 percent and with the indirect hemagglutination reaction, we found the plague microbe in 39.6 percent of the suspensions. In 31 suspensions that gave a positive bacteriological result, antigens of the plague microbe were found only in the enzyme-marked antibody reaction and not in the indirect hemagglutination reaction.

In an examination under field conditions of material from 12 biological test animals that died, the carcasses of 7 wild rodents, 3 camels, and 15 group suspensions of ectoparasistes, cultures of the plague microbe were isolated from 9 of the biological test animals and from 1 steppe lemming. In 8 of the suspensions of internal organs of the biological test animals and in the suspension taken from the steppe lemming, antigens of the plague microbe were found in the enzyme-marked antibody reaction and in the indirect hemagglutination reaction.

Thus, the enzyme-marked antibody reaction is more sensitive than the indirect hemagglutination reaction and can be used in epizootological surveys of natural plague nidi.

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EVALUATING POSSIBILITY OF INCREASING IMMUNOGENICITY OF EV STRAIN USING INDUCED MUTATIONS METHOD

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[Article by O. Ya. Aymanova (Alma-Ata)]

[Text] The search for an effective vaccine against plague is still an urgent task.

In addition to obtaining new vaccine strains, there is another path toward achieving this goal that can be pursued--increasing the immunogenicity of existing vaccines.

We have obtained a large group of auxotrophic mutants from the EV vaccine strain using the method proposed earlier that utilizes the effect of nitrosoguanidine (Stepanov and Kondrat'yeva, 1974). In terms of their microbiological properties these mutants did not differ from the original strain used to produce the live plague vaccine. To study their immunogenicity, we chose 1 mutant for each experiment that was dependent on arginine, on arginine and glutamic acid, and on proline and glycine.

The immunogenic properties of the mutants were studied in accordance with the criteria for selecting plague microbe vaccine strains (Saratov, 1974). The minimum immunizing dose was determined in experiments on white mice and guinea pigs. The guinea pigs were immunized subcutaneously in doses of 10^2, $5 \cdot 10^2$, and $2.5 \cdot 10^3$ microbial cells from a 48-hour culture of the original strain and mutants, with 5 animals per dose. White mice were immunized with the following doses: $5 \cdot 10^2$, $2.5 \cdot 10^3$, $1.25 \cdot 10^4$, $6.25 \cdot 10^4$ microbial cells, with 10 animals per dose. After 21 days, during which no death among the experimental animals was noted, a control infection was applied using 200 Dcl of the virulent standard strain Yersinia pestis 231.

The immunogenicity of the orginal strain and the mutants was described by the ED_{50} value. For the original strain this value was 426 microbial cells for guinea pigs and 17,780 microbial cells for white mice.

A necessary condition for the immunogenicity of the original strain is its ability to take hold and multiply in the organism's tissues, which is called residual virulence (Korobkova, 1956).

To determine the extent and rate of the microbes' spread throughout the organs and tissues, guinea pigs were immunized subcutaneously in doses of 10^2, $5 \cdot 10^2$, 10^6, $2 \cdot 10^9$, and $15 \cdot 10^9$ microbial cells, with 30 animals per dose. Then 3 animals each were killed 1, 3, 5, 7, 14, and 30 days following the introduction of the cultures.

The data we obtained showed that the arginine-dependent mutants of the EV strain had the highest rate of survival; this apparently explains the significantly higher immunogenicity of this mutant compared to the original EV strain. With high doses ($15 \cdot 10^9$ microbial cells) the mutant penetrated into the blood and bone marrow.

The results we obtained allow us to believe that there are many prospects for further, in-depth research on the immunogenicity and harmlessness of arginine- and glutamine-dependent mutants of the EV strain.

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AUTOANTIBODY FORMATION IN BRUCELLOSIS

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[Article by N. I. Tikhenko, I. F. Taran, and M. N. Nurmagomedov (Stavropol)]

[Text] Allergic reactions to microorganisms and various antibodies play a significant role in the pathogenesis of brucellosis. It has been established that in brucellosis (Tatishvili, 1969; Musabayev, Nikolayev, Nazarmukhamedov, 1973; Tikhenko and Taran, 1978; etc.) and in other diseases (Borisov, 1966; Saidov, 1971; Shanina and Dal'vadyants, 1978), processes of autosensitization with the formation of the corresponding autoantibodies are of definite pathogenic importance. The existence of autoantibodies and their involvement in the disease make it necessary to explain processes such as the degree of participation of autoantibodies in the pathological process and their role in the formation of central and other lesions in organs and tissues.

In animals infected with Brucella abortus 544, B. melitensis 565, and B. suis 1330, the titers of autoantibodies were significantly higher than in healthy animals over the course of 6 months following the infection. In animals infected by an agent of cattle brucellosis, the titers of autoantibodies were lower than in animals infected with an agent of brucellosis from sheep, goats, and pigs. In all the animals infected with a brucellosis agent, the highest titers of autoantibodies were against antigens from the spleen and lymph nodes. The titers of autoantibodies against antigens from the liver and heart were significantly lower. The maximum titers of antibodies were reached 30 days after the infection, followed by a gradual decline; the highest titers of autoantibodies were recorded after 2-2.5 months. The drop in the titers of autoantibodies was less marked than that seen in the titers of specific antibodies.

In animals infected with brucellosis agents from sheep, goats, and especially pigs, the titers of autoantibodies against antigens from the spleen and lymph nodes were significantly higher than those seen in animals infected with B. abortus. Significant differences were seen as early as the fifteenth day in autoantibodies against the spleen antigen. After 2.5-3 months the titers of autoantibodies in guinea pigs infected with B. abortus did not differ substantially from the titers found in guinea pigs infected with B. melitensis. In animals infected with B. suis 1330, after 120 days the titers of autoantibodies were significantly higher than in those animals infected with B.

abortus. A similar pattern of autoantibody formation, although less pronounced, was seen in the formation of autoantibodies against lymph node antigens.

The most marked responses to Burne's intracutaneous allergic test in all the groups of animals were seen 60-75 days following infection. In animals infected with B. abortus the average area of infiltration when the brucellosis was introduced was 2 x 2 cm; in the animals infected with B. melitensis and B. suis, the average area was between 3 x 3 cm and 3 x 4 cm. In the later stages of the reaction to the allergen, the area decreased somewhat. The most marked allergic response in the organisms of infected animals was accompanied by intensive formation of autoantibodies.

It is known that in guinea pigs the greatest generalization of the infection process occurs by the end of the first month following infection. This generalization is accompanied by the most pronounced injury caused by the agent and its endotoxin on the cells and tissues of the organism. This is confirmed by the appearance of granules and areas of necrosis in various organs and tissues in the presence of a brucellosis infection (Taran, 1967). Naturally, cellular and humoral defense mechanisms are activated in this case in the form of accumulation of autoantibodies, the development of a pronounced macrophage reaction, and sensitization of the organism. Correlations between the activity of autoantibody formation and an increase in the organism's sensitivity status indicate that there is a tie between autoimmune processes and allergic development in the organism, which also probably determines pathogenic characteristics of the brucellosis infection. At various stages of development of the pathological process, it is possible for the autoantibodies to cause injury, that is, they can participate in the development of the pathological process. It is known that a number of investigators (Berezhnaya, 1967; Movet, 1975; Shevelev, 1978; etc.) succeeded in reproducing various pathological states using cytotoxic sera and several other methods to induce autoimmune responses.

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USING CHICKEN YOLK TO STUDY IMMUNOGENICITY OF CHOLERA VACCINES

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[Article by E. S. Karetnikova, S. N. Barakhovskaya, and V. L. Chugay (Irkutsk)]

[Text] Due to the lack of an experimental model for testing the protective activity of cholera vaccines, usually the sensitivity of experimental animals to the infection is artificially increased. Recommendations have been made to use chicken yolk, mucin, or ferrous ammonium citrate for this purpose (Yefremova and Kolesnik, 1962; Karayeva et al., 1976; Feeley and Pittman, 1965; Ford and Hayhoe (1976).

Of the preparations that have been suggested, the most widely used is commercial mucin made in the United States and England, as well as mucin prepared in laboratories. It is somewhat difficult to obtain commercial mucin, and furthermore, it is not always sufficiently active. When preparing mucin under laboratory conditions, small series of the preparation are obtained that lack standardized activity.

Yefremova and Kolesnik developed a process for increasing the sensitivity of animals to cholera infection by introducing a culture with chicken yolk, but no methods have been worked out that can be used in the contemporary testing of the protective activity of cholera vaccines (the testing method described by Feeley and Pittman).

We attempted to study the possibility of replacing mucin with chicken yolk in testing the defense activity of cholera corpuscular vaccine and cholerogen-anatoxin.

We used infectious and industrial strains of cholera v. eltor (P-3122, M-879, P-3116, and M-878).

We tested the virulence of these cultures and the protective activity of the cholera vaccines according to the methods recommended in the technical specifications for the cholera corpuscular vaccine and the cholerogen-anatoxin (regulation No 115-78, TU 42.14 No 61-78, regulation No 34-75, TU 42.14 37-75); we used mixed breed white mice weighing 16-18 g and 10-12 g. The virulence of the vibrions was judged on the basis of the LD_{50} value. The protective

activity of the cholera vaccines was evaluated on the basis of the ED_{50} value and the antigenicity coefficient.

We determined the virulence of the cholera vibrions when chicken yolk was used in experiments that were repeated numerous times. In each experiment as a control we infected the same number of animals with a culture suspended in mucin or in an isotonic NaCl solution. Animals in two groups (10 mice in each) were given either yolk or mucin without any of the culture.

The protective activity was studied for 3 series of corpuscular cholera vaccine and 10 series of cholerogen-anatoxin. The protective activity of commercial cholera vaccine was tested in parallel with a study of a national reference preparation.

In the experiments we used a 3-hour agar culture of cholera vibrions, from which we prepared 2-fold dilutions in an isotonic NaCl solution; then from the dilutions we took 0.05 ml of the culture and placed it in 0.45 ml suspensions of yolk, mucin, and an isotonic NaCl solution. To determine the LD_{50} value we used 6 groups of animals (5 animals in each group) and introduced the culture in doses ranging from 94 to 3000 microbial cells.

We prepared the yolk immediately before performing the experiments or we used freeze-dried preparations. Following aseptic procedures, we separated the native yolk from the white and suspended it in 7 ml of an isotonic NaCl solution. We determined the pH of the yolk suspension (it was usually 6.5) and brought it to 8.0 by adding a 20 percent NaOH solution. After this the yolk suspension was filtered through sterile gauze and was then used for the infection. This same suspension was dried using a sublimation method.

In the experiments we also used a 5 percent suspension of mucin, consisting of equal quantities of the American and English commercial preparations.

These tests established an increase in the sensitivity of white mice to infection with a culture of cholera vibrion suspended in chicken yolk. When the mice were given a suspension of vibrions in an isotonic NaCl solution in doses ranging from 94 to 3000 microbial cells, as a rule none of the animals died. Infecting white mice with cultures containing the same doses of vibrions suspended in mucin and chicken yolk resulted in the death of a significant number of experimental animals: the LD_{50} value when mucin was used was 11.1 ± 5.5, and when native chicken yolk with a pH of 8.0 was used, the LD_{50} was 20.1 ± 10.1. When we used a culture with yolk and the pH had not been brought up to 8.0, the LD_{50} was significantly higher and could reach 1000-2000 microbial cells. Freeze-dried preparations of yolk were less active: the LD_{50} when these preparations were used was between 3000 and 3500 microbial cells.

Control suspensions of mucin and yolk (not containing cholera vibrions) introduced intra-abdominally did not cause death among the white mice.

When we tested the protective activity of the cholera corpuscular vaccine using a suspension of infectious cultures of native chicken yolk (pH 8.0) as a medium, the ED_{50} for white mice given preparations of different series ranged from $5 \cdot 10^5$ to $2.03 \cdot 10^6$ microbial cells. The ED_{50} of the reference vaccine in

the same experiments was between $1.01 \cdot 10^6$ and $2.8 \cdot 10^6$ microbial cells. The coefficient of antigenicity of the cholera corpuscular vaccine ranged between 0.7 and 2.5; that is, in every case it exceeded the minimum indicators outlined in TU 42.14. In the control group of unvaccinated animals (10 mice) when the culture was introduced in doses ranging from 137 to 913 LD_{50}, 7-10 mice died.

The ED_{50} for the cholerogen anatoxin of various series when immunized animals were infected with virulent cultures of the cholera vibrion suspended in chicken yolk ranged from 0.11 to 1.02 μg. In the control group 7-10 animals died.

The results we obtained indicate that when using native yolk as a suspension medium for cholera vibrions for the purpose of infecting white mice, indicators of the cultures' virulence and the immunogenicity of the cholera vaccines meet the requirements set down in the technical specifications for the cholera corpuscular vaccine and cholerogen anatoxin. Native chicken yolk and the mucin recommended in TU 42.14 are equivalent in terms of their ability to increase the sensitivity of laboratory animals to cholera vibrions. Therefore, when determining the degree of virulence of cholera vibrion cultures and the protective activity of cholera vaccine, native chicken yolk can be used as a suspension medium after its pH has been brought up to 8.0.

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DETERMINING EPIDEMIC POTENTIAL OF A NATURAL NIDUS SECTION

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[Article by N. I. Khot'ko, Ye. V. Kuklev, and I. S. Soldatkin (Saratov)]

[Text] The method that we developed (Kuklev et al., 1981) for making a quantitative evaluation of the epidemic potential of a natural plague nidus can also be applied to other infections that have natural nidi. It is based on 3 elements that determine the size of the potential: the presence and extent of epizootics, the degree of contact between the human population and the natural nidus, and the social factor. The simplicity of the evaluation and the substantial reduction in subjectivity it provides mean that this method can be used widely in epidemiological mapping of natural nidi of various infections. In order to do this one must obtain a description of quite small sections of the nidus and reflect them in the map. These sections can be divided up according to some formal system (such as a grid of quadrants) or according to some independent feature.

Here a number of questions arise that are in need of methodological resolution. Below we discuss the primary questions involved.

The selection of the size of the section and the method for separating it from the rest affects both methodology and the practicality of carrying it out. In our opinion, it is preferable to use a formal division of the territory. It is practical to used 20-kilometer quadrants in a grid of "primary regions" when working with natural plague nidi (Methodological Recommendations for Certification of Natural Plague Nidi in the USSR, Moscow, 1977). These divisions provide sufficient detail and availability of the appropirate information (at least for the majority of nidi located in flat areas).

A point-system for evaluating the presence and extent of epizootics should include the factor of time. The evaluation of the epidemic potential of an entire nidus applies to a certain season, since it takes into account the epizootic conditions which on a nidus-wide scale are correct only for a specific season. On the scale of an individual section of the nidus, especially when the section is small, the epizootic conditions can change very rapidly and it is obviously necessary to take into account the duration of the epizootic. Furthermore, compilation of an epidemiological map assumes that

long-term characteristics of the sections in the territory are fixed. Therefore, an evaluation of the area of an epizootic is at some point in time incorrect. It is also important to note that given the actual intensity of survey work, a correct evaluation of the epizootic conditions can be given only for fairly large sections.

Taking all this into account, the points given to describe the intensity of epizootic conditions should be determined in the following sequence. First, using the body of data from several previous years, one should determine the average incidence of epizootics (and assign the appropriate number of points) in a section that includes the 9-10 primary regions. In natural nidi where epizootological divisions have been made, this section is usually separated as a "landscape-ecological region." Then each primary region is assigned a certain number of points. The basis for these evaluations is not direct data on epizootics (on the scale of primary regions they are usually not complete enough), but rather information on the nature of populations of plague carriers, their size, the abundance of ectoparasites, and so on. The final stage is the control stage--a comparison of the arithmetic mean of the evaluations of individual primary regions and the evaluation of the entire territory of the nidus. When there are discrepancies the appropriate corrections are made in the evaluations of the primary regions.

The second element of the epidemic potential is a point-system evaluation of the human population's contact with the natural nidus; it is determined for a primary region using the same methods that are used for the entire nidus. However, some characteristics that depend on the small size of the section being evaluated stand out. For example, it becomes necessary to calculate the number of people who come into contact with the nidus. When the nidus as a whole is being evaulated this factor also has an effect; however, it is completely acceptable to consider the size of the population as a constant value. This is not the case when evaluating a section of the nidus. In the first place, neighboring sections with approximately the same epizootological characteristics may have significant differences in terms of the number of people coming into contact with the nidus. In the second place, conditions may be created in which there are temporary concentrations of people in certain sites (such as seasonal work). When the seasonal work period coincides with the time at which the nidus is activated, the degree of contact naturally increases significanty. There is a corresponding increase in the number of points given to that particular section. Therefore, determining the second element of the epidemic potential of a section of the nidus requires a more detailed examination of the characteristics of the population's economic activities.

The third element of the epidemic potential describes the conditions under which the infection is manifested or spread among humans. The social factor is not uniform; it is inaccurate for small sections and it must be averaged for the entire nidus. Furthermore, the importance of the social factor should be viewed differently; in our case, it should be considered from the position of the present, that is, taking into account specific manifestations of the epidemic process under given social relations. In light of this, it is clear that the social factor is a value that changes relatively slowly; therefore, in compiling an epidemiological map it is wise to use that component of the social

factor that can play a decisive role in spreading infection, and can consequently influence the number of points given to that element in particular and to the epidemic potential as a whole. When medical aspects are involved, for example, this component could be the level of medical service; the system of preventive measures; the results of epidemiological, bacteriological, and serological research. When economic aspects are being considered, this component could be the degree and character of the territory's development which determine the ways in which the population comes into contact with natural preconditions of infections, and so forth.

The accumulation of additional material will permit us to determine the characteristics that will make it possible to move from a general certification evaluation to a more detailed evaluation of the social factor.

In conclusion, we should examine one more aspect of the question--the relationship between the epidemic potential of an entire nidus and the evaluations of the epidemic potential of individual sections. In spite of the fundamental similarity among these characteristics, we cannot require that one description be translated into the other because of a number of logical and mathematical considerations. This requirement is fair with regard to individual elements of the epidemic potential; but an average of the products of the points describing each section does not necessarily correspond to the product of the averaged points. The logical aspect of the incomplete compatibility of these concepts lies in the fact the evaluations of the epidemic potential of an entire nidus and of its individual sections are made for different reasons.

Epidemiological mapping taking into account the epidemic potential of natural nidi of infections and their sections will make it possible to evaluate and predict the danger of epidemics and to divide a territory up into epidemiological regions. The accumulation and generalization of materials are not only important stages in planning further scientific studies, they also make it possible through purposeful and economical implementation of preventive measures to arrive at a theoretical and practical program for making radical improvements in natural nidi (as sections of a locality) by transforming them and cultivating them.

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